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CLONING AND CHARACTERIZATION OF THE CD3 eta SUBUNIT

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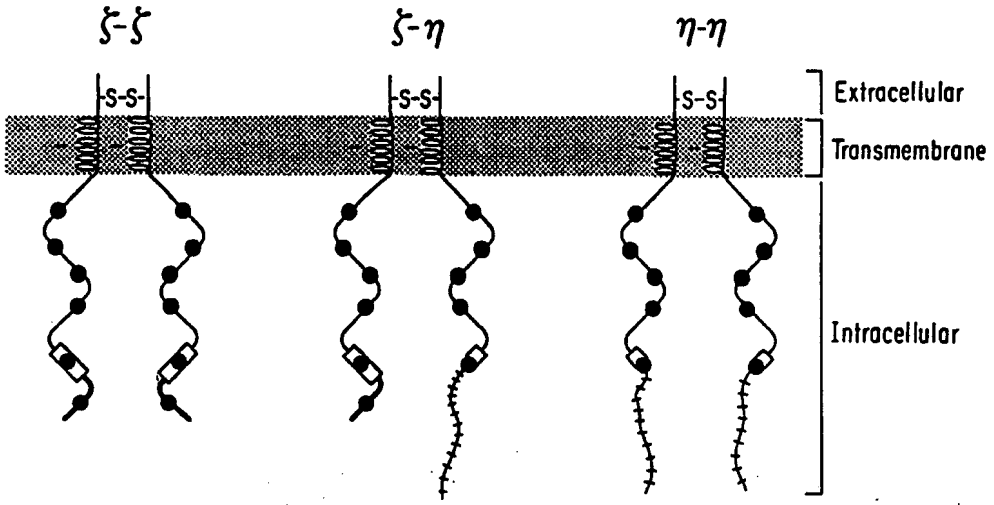

(57) Abstract

Primary structure of CD3η of eukaryotic origin is deduced and is compared with the structure of CD3ζ with which it forms a heterodimer.

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CLONING AND CHARACTERIZATION OF THE CD3η SUBUNIT

Background of the Invention

The T cell receptor (TCR) is a molecular complex consisting of multiple subunits that mediate the recognition of antigen in the context of a particular major histocompatibility complex (MHC) product. Meuer, S.C., et al., Ann. Rev. Immunol. 2:23-50 (1984); Clevers, H., et al., Ann. Rev. Immunol. 6:629-662 (1988); Davis, M.M. and P.J. Bjorkman, Nature 334:395-402 (1988). The antigen/MHC binding moiety, termed Ti, is a disulfide-linked heterodimer consisting of one α and one β subunit on the vast majority of peripheral T lymphocytes. Both subunits are immunoglobulin-like being composed of variable and constant domains, the former encoding the unique specificity of a given T cell clone. Ti, in turn, is non-covalently associated with a set of invariant subunits, collectively termed CD3, involved in signal transduction. CD3γ, δ and ϵ subunits are encoded by related genes that are closely linked. In addition, a fourth CD3 subunit present in all T cell receptors is the 16KD CD3ζ subunit which is structurally and genetically distinct from the other invariant chains and exists predominantly as a 32KD homodimer within the TCR complex. Weissman, A.M., et al., Science 239:1018-1021 (1988); Baniyash, M., et al., J. Biol. Chem. 264:13252-13257 (1989). The important contribution of CD3ζ to T cell receptor mediated activation has been demonstrated by analysis of CD3ζ mutants derived from antigen specific MHC-restricted T-T hybridomas. Sussman, J.J., et al., Cell 52:85-95 (1988). Loss of

CD3ζ expression leads to virtual abrogation of IL-2 production upon stimulation by antigen and MHC.

Recently, it has been demonstrated that CD3ζ can be complexed in a heterodimeric form with a 22KD subunit termed CD3η. Baniyash, M., et al., J. Biol. Chem. 263:9874-9878 (1988). Despite the fact that the copy number of TCR-associated CD3ζ-η heterodimers is one order of magnitude less than CD3ζ-ζ homodimers in antigen-specific T cell hybridomas, recent evidence suggests that the CD3ζ-η structure is important in coupling the TCR to phosphoinositide hydrolysis. Mercep, M, et al., Science 242:571-574 (1988). Other studies have suggested that CD3ζ-η may be linked to antigen-induced cell death in these hybridomas. Mercep, M, et al., Science 246:1162-1165 (1989). At present, there is virtually no definitive structural information concerning CD3η, except that it is not a post-translational modification of CD3ζ resulting from phosphorylation, glycosylation or sulfation. Orloff, D.G., et al., J. Biol. Chem. 264:14812-14817 (1989). Although amino acid analysis by metabolic labeling has shown clearcut differences between CD3η and CD3ζ, certain anti-CD3ζ peptide antisera react with CD3η, implying a structural relatedness. Orloff, D.G., et al., J. Biol. Chem. 264:14812-14817 (1989).

Summary of the Invention

The present invention relates to CD3η of eukaryotic origin, which is known to combine with CD3ζ to form a CD3ζ-η heterodimer which is important in coupling the T cell receptor to phosphoinositide hydrolysis and appears to play a role in antigen-

induced cell death. For the first time, there is evidence for the existence of a CD3η-η homodimer. As described herein, there is substantially greater expression of CD3η in immature tissue (2 week-old thymus) than in mature spleen, suggesting that the CD3ζ-η and the CD3η-η dimers are expressed differentially during thymocyte development.

As is also described herein, the primary structure (amino acid sequence) of eukaryotic CD3η (specifically murine CD3η) has been determined, using protein microsequencing and cDNA cloning, and compared with that of CD3ζ, demonstrating that the amino-terminal sequences of the two are identical through the first 122 amino acids and that the amino acid sequences of the carboxy-terminal regions (cytoplasmic tails) of the two are divergent. As further described herein, it has been shown that the message for the two types of subunits is expressed preferentially in different tissues. That is, the message for CD3η is expressed preferentially in thymocytes (thymic lymphocytes), relative to splenic T lymphocytes.

The work described herein supports the idea that either the CD3η-η dimer or the CD3ζ-η dimer is responsible for linking T cell receptors on thymocytes to different cellular pathways than the cellular pathway(s) to which the CD3ζ-ζ homodimer links T cell receptors in mature T cells.

The present invention further relates to soluble proteins having amino acid sequences that correspond to the extracellular or cytoplasmic domain of CD3η, to reagents, such as oligonucleotide probes and antibodies, useful in the detection of CD3η in tissue and

to methods for screening substances which are agonistic or antagonistic to CD3-mediated responses. As a result of the present invention, it is possible to further characterize CD3η, the dimers of which it is a constituent and its role in T cell receptor activation and possible T cell development. This is particularly valuable because of the role of CD3η in the hydrolysis of phosphatidylinositol biphosphate to inositol triphosphate (Ins P_3) and 1,2-diacylglycerol (DAG), which appear to be second messengers which transmit signals for initiation of later events, such as secretion of lymphokines and expression of lymphokine receptors.

Brief Description of the Drawings

Figure 1 shows the cDNA and predicted protein sequences of clone pBS17.

Figure 2 shows a protein sequence alignment of mature murine CD3ζ and CD3η by the genetic code matrix analysis. Open gap cost and unit cap cost were each set at 100. Identity value = 85.3%. Identity of amino acids in the two sequences is marked. Beyond amino acid 122 the sequences are divergent.

Figure 3 shows the in vitro translation of CD3η, lane 1, human CD3ζ; lane 2, murine CD3η; lane 3, no added RNA; lane 4, Bovine Pappiloma virus (BPV) RNA. Molecular weight markers and their relative mobilities (in parentheses) are as follows: 97 (94) KD, phosphorylase B; 68 (68) KD, Bovine Serum albumin (BSA); 45 (45) KD OVA; 31 (29) KD, carbonic anhydrase; 22 (20) KD, soybean trypsin inhibitor and 14 (14) KD, lysozyme.

Figure 4 is a model of putative CD3ζ-η heterodimers and CD3ζ-ζ and CD3η-η homodimers.

Figure 5 is a two dimensional diagonal sodium dodecasulfate-polyacrylamide gel electrophoresis ($NaDodSO_4$-PAGE) analysis of digitonin lysates of ^{125}I-labeled murine 3DO54.8 T cell immunoprecipitated by anti-CD3ϵ monoclonal antibody (MAb). Molecular weight standards are as in Figure 3.

Figure 6 shows an RNA blot analysis of CD3η expression. A) 10μg of total RNA from 2 week old Balb/c thymuses, Balb/c spleens and the murine hybridoma 3DO54.8. B) 5μg of polyA+RNA from murine spleens, the hybridoma 3DO54.8, cell lines EL4 (T cell lymphoma). CTLL (T cell), A20 and M12.1.4. (B cell lymphomas) and murine livers.

Figure 7 shows the organization of exons 2-9 of the CD3$\zeta\eta$ genomic locus. Exons are indicated with black boxes. BamHI (B) and HindIII (H) sites are indicated by long and short vertical lines, respectively. The dashed line 3' to exon 8 and 5' to the downstream HindIII site indicates variation in size of this fragment in recombinant phage. SA and LA = short arm and long arm of the λEMBL3 phage, respectively. The alternative splicing patterns of exons 1-9 giving rise to the CD3ζ and CD3η mRNAs are shown below the phage maps.

Figure 8 shows the sequence of CD3η-specific exon 9 and flanking regions. Flanking regions are indicated by lower case letters including the canonical splice acceptor site 5' to exon 9. Exon 9 encodes the final 63 aa of the CD3η protein and the entire 3' UTR of the CD3η mRNA. The polyadenylation site is underlined.

Figure 9 is a schematic representation of the CD3η cDNA clone pBS17 and its derivative pBSΔ17. Exon 9

specific sequence (black bold line) and the CD3η specific 5'UTR (positions 1-34) are indicated. The specific hybridization of CD3ζ and CD3η mRNA to the antisense RNA probe synthesized from pBSΔ17 is also indicated. Upon single strand specific RNase digestion, CD3ζ and CD3η mRNA protect 100 and 173 bases of the hybridized probe, respectively.

Detailed Description of the Invention

The present invention is based on sequencing and characterization of CD3η of eukaryotic origin, which has recently been shown to be present in heterodimers with CD3ζ, one of the four subunits present in all T cell receptors. As described below, it has now been shown that CD3$\zeta$and CD3η exhibit structural similarities, which are limited to the two amino-terminal regions, and structural differences, present in the third or carboxy-terminal region. The differences observed provide a structural basis for biologically distinct differences between CD3η and CD3ζ function. One such difference (i.e., the occurrence of a nucleotide binding site within CD3$\zeta$and its alteration in CD3η) could result in the two being differentially regulated by exogenous signals and/or being involved in mediating different functional effects.

As is also described, there is now evidence that CD3η also exists as a CD3η-η which is preferentially expressed in thymocytes, rather than on mature, splenic T cells. This preferential expression suggests that CD3η-η or CD3ζ-η dimers are responsible for linking T cell receptors on thymocytes to different cellular pathways than those to which CD3ζ-ζ dimers link T cell

receptors in mature T cells. It is possible that programmed cell death of thymocytes which results from crosslinking of the receptor is mediated by CD3η, with the result that CD3η would have an important role in thymocyte selection.

The following is a description of cloning and sequencing of the CD3η subunit, of comparison of CD3η and CD3ζ structure and expression and of the role of CD3η in T cell receptor activation.

The CD3η subunit of the T cell receptor forms a heterodimeric structure with CD3ζ in thymus-derived lymphoid cells and is apparently involved in signal transduction through the TCR. The primary structure of murine CD3η was deduced from protein microsequencing and cDNA cloning and is shown in Figure 1. The mature protein is divided into three domains: a 9 amino acid (aa) extracellular segment, a 21 amino acid transmembrane segment including a negatively charged residue characteristic of CD3 subunits, and a 155 amino acid cytoplasmic tail. The amino-terminal sequences of CD3η and CD3ζ are identical through amino acid 122 of each mature protein but then diverge in the remainder of their respective carboxy-terminal regions, consistent with alternatively spliced products of a common gene. The cytoplasmic domain of CD3η is 42 amino acid larger than that of CD3ζ but lacks one of six potential tyrosine phosphorylation sites as well as a putative nucleotide binding site previously identified in CD3ζ (Weismann, A.M. et al. Science 239:1018-1021 (1988)). These structural features presumably account for the difference between CD3η and CD3ζ function. Furthermore, northern analysis demonstrates preferential

expression of CD3η message in thymocytes relative to splenic T lymphocytes, consistent with the notion that CD3η may be an important component of a TCR isoform(s) during thymic development.

Stimulation of the CD3 receptor results in the hydrolysis of phosphoinositides to water-soluble inositol phosphates (IP). Phosphoinositide hydrolysis occurs during early stages of T cell activation and also results in the production of diacylglycerol (DAG) which is an endogenous activator of protein kinase C. Such production has been shown to play a role in activation-induced phosphorylation of the CD3γ chain. These events play a critical role in T lymphocyte activation. In particular, it has been shown that loss of CD3ζ expression abrogates phosphoinositide hydrolysis and subsequent IL-2 production upon stimulation by antigen (Mercep, M. et al., Science 242:571-574 (1988). This suggests that the CD3η subunit may be important for linking T cell receptors on thymocytes to different cellular pathways than those associated with CD3ζ-ζ.

Based on the sequencing of CD3η subunit described herein, isolated CD3η T lymphocyte surface receptor subunit DNA or fragment thereof having all or a portion of the nucleotide sequence shown in Figure 1 or a functional equivalent thereof can be produced. Preferably, the DNA is of rodent or human origin and encodes all or a portion of the cytoplasmic domain of CD3η. A functional equivalent is any nucleic acid sequence which encodes a product having essentially the same characteristics and functions as that encoded by the DNA of Figure 1. In addition, the native DNA sequence

of CD3η can be modified by deletion, insertion or substitution of nucleotides to yield products which exhibit substantially the same functional properties as those of native CD3η subunit. The DNA sequences can be chemically synthesized or they can be obtained from natural sources using recombinant DNA technology.

Soluble portions of the CD3η subunit can also be made which correspond to the extracellular or cytoplasmic domain of CD3η or fragment of such domain. A portion of the transmembrane domain may be included; the portion will be of such a size that it does not substantially affect the solubility of the CD3η subunit. The soluble CD3η subunits can have the amino acid sequence depicted in Figure 1 or the functional equivalent thereof. Soluble CD3η subunits can be made by enzymatic fragmentation, peptide synthesis or recombinant technology. They can be used to inhibit T lymphocyte function, particularly the cytoplasmic domain or portion thereof which is involved in signal transduction of CD3-mediated T lymphocyte activation signal into a cell.

This invention also pertains to cells which have been transformed to express CD3η protein on its surface. The cells are derived from cells which do not normally express CD3η protein or do not express the CD3η protein at significant levels on its surface. Preferably, a continuous non-CD3 bearing cell line is transformed with an expression vector containing CD3-encoding DNA to thereby cause the cell line to express CD3η protein on its surface. Typically, the expression vector will contain the DNA sequence encoding the extracellular, transmembrane and cytoplasmic

domains of human CD3η glycoprotein. Preferably, the transformed cells will express all or a portion of the cytoplasmic domain of CD3η which is necessary for transduction of a CD3-mediated T lymphocyte activation signal into a cell (particularly the carboxy terminal portion of the cytoplasmic which is not homologous to the carboxy terminus of CD3ζ).

Relatedness of CD3η and CD3ζ Sequences

Comparison of the predicted mature murine CD3η and ζ sequences by gene matrix analysis is given in Fig. 2. As shown, there is an identity of the amino-terminal 122 amino acid (aa) of both proteins. In addition, the leader sequences are identical. However, the carboxy-terminal sequences of CD3ζ and η are entirely divergent. Thus, both proteins are identical in the extracellular and transmembrane segments but diverge in the cytoplasmic tails resulting in CD3ζ and η cytoplasmic domains of 113 and 155 amino acids, respectively. Comparison of the amino acid residues in predicted CD3ζ and η sequences agrees well with empirically derived amino acid analysis using metabolically labeled cell lysates (Orloff, D.G., et al., J. Biol. Chem. 264:14812-14817 (1989)), particularly in the presence of two tryptophan residues in CD3η sequence which are lacking from CD3ζ and an additional cysteine residue in the CD3η sequence. Computer search of GenBank 61, EMBL 20, PIR 21-0 and Swiss-Protll data bases utilizing the CD3η specific DNA and protein sequences failed to uncover any significant matches with known structures.

Given the amino-terminal identity and carboxy-terminal divergence of CD3ζ and η sequences, it is likely that CD3η is an alternatively spliced form of a gene common to both CD3η and ζ. The region of CD3η divergence from the published CD3ζ DNA sequence follows that segment encoded by exon 7 at base pair (bp) 568, one bp beyond the splice junction at bp 567. An identical junctional sequence was found in one additional independent clone identified by rescreening the library with a CD3η specific probe (bp 633-1362, pBS17). It is therefore likely that CD3ζ exon 8 has been spliced out from the pre-mRNA and replaced by a downstream exon encoding the unique CD3η sequence which also has a G nucleotide at the 5' end of the exon. The CD3η exon does not appear to be within the intron between CD3ζ exons 7 and 8 because a polymerase chain reaction (PCR) product derived from priming DNA with amplimers corresponding to CD3ζ exon 7 (sense) and ζ exon 8 (anti-sense) sequences did not hybridize with an CD3η-specific cDNA clone. There is also the possibility of 5' alternative splicing given the divergence between CD3ζ and η nucleotide sequences noted in the 5' untranslated region of the CD3η cDNA 5' of bp 35 (Fig. 3) and the published flanking sequence of the murine genomic CD3ζ clone. Baniyash, M., J. Biol. Chem., 264:13252-13257 (1988). For these reasons, the possibility that CD3η and CD3ζ arose from gene duplication is more remote, although it is not yet formally excluded.

In Vitro Translation of Synthetic Murine η RNA

Open reading frame analysis of the CD3η cDNA sequence predicts a molecular weight (MW) of 23,339 daltons for pre-CD3η. In vitro transcription of the pBS17 cDNA insert with T7 RNA polymerase yielded synthetic RNA with an apparent size of 1.5Kb in 1.2% formaldehyde-MOPS agarose gel electrophoresis. Translation of this RNA in vitro resulted in a major protein product of MW 23KB as shown in Fig. 3, lane 2. The size of this major band is in excellent agreement with the predicted molecular weight of pre-CD3η. The minor bands at 24KD and 16KD may result from translation of endogenous RNA or premature termination of RNA translation. Hames, B.D. and S.J. Higgins, (eds), Transcription and Translation: A Practical Approach (IRL Press, Oxford, England) (1986). Synthetic RNA from the human CD3ζ encoding clone pGEM3Z-CD3ζ produced a major protein product of 17.8KD (Fig. 3, lane 1), in close agreement with the predicted molecular weight of 18,608 for pre-CD3ζ while Bovine Papilloma Virus RNA gave the expected pattern (Fig. 3, lane 4). Translation in the absence of added RNA gave no detectable protein (Fig. 3, lane 3).

Analysis of cellular variants expressing differential levels of CD3η has led to the notion that CD3η expression may be important and perhaps necessary for phosphoinositide turnover. Mercep, M., et al., Science 242:571-574 (1988). Furthermore, TCR crosslinking which results in cell cycle arrest of T cell hybridomas is linked to the level of CD3η expression. Mercep. M., et al., Science 246:1162-1165 (1989).

The cloning of CD3η as reported herein provides a definitive structural basis for biologically distinct differences between CD3η and ζ function. The model of putative CD3ζ-η heterodimers and CD3ζ-ζ and CD3η-η homodimers is shown in Fig. 4. The single negatively charged (-) Asp (at amino acid +15) in the transmembrane region is shown. Try residues (O) are indicated as is the boxed putative nucleotide binding site in the cytoplasmic domain (□). CD3ζ and η specific protein stretches are highlighted by dark solid and hatched lines, respectively.

As shown in Fig. 4, CD3ζ possesses six cytoplasmic tyrosine residues whereas CD3η has only five. Given that CD3ζ but not CD3η has been reported to be phosphorylated on tyrosine residues after TCR triggering (Baniyash, M., et al., J. Biol. Chem. 263:18225-18230 (1988)), the Tyr at position 132 may have a regulartory role. Others have construed that the sequence GxGxxGxxxGxxxAxK within the CD3ζ sequence (amino acids 114-129) may represent a nucleotide binding site. Weissman, A.M., et al., Science 239: 1018-1021 (1988). However, nucleotide binding sites have been shown to occur at the junction between a β sheet and α helical segment and as such, are preceded by and include hydrophobic residues. Wierenga, R.K. and W.G.J. Hol, Nature, 302:842-844 (1983); Moller, W. and R. Amons, FEBS Lett., 186:1-6 (1985). This is not the case for CD3ζ where the putative site is preceded by the four charged residues ERRR. Nevertheless, if a di- or trinucleotide binding site exists within the CD3ζ sequence at positions 114-129, it is almost certain to be altered in CD3η since that putative motif has been

disrupted within the CD3η sequence after the third glycine residue. How this difference will affect the functions of CD3η and ζ remains to be shown, but could result in CD3ζ and η being differentially regulated by exogenous signals and/or themselves mediating different functional effects.

Figure 4 also raises the possibility that three dimeric forms of CD3ζ and/or η exist: CD3ζ-ζ homodimers; CD3ζ-η heterodimers; or CD3η-η homodimers. In view of the northern data indicating a substantially greater expression of CD3η in 2 week old thymus as opposed to spleen, the possibility that CD3ζ-η or CD3η-η dimers are differentially expressed during thymocyte development must be considered. That the latter has not been previously observed may be consequence of the low level of CD3η expression and a present lack of CD3η specific reagents. Analysis of murine CD3η and ζ RNA expression during gestation, as well as development of CD3η specific antibodies to examine protein levels in individual cells will help resolve these issues. One must seriously entertain the possibility that either CD3η-η or CD3ζ-η dimers may be responsible for linking T cell receptors on thymocytes to different cellular pathways than CD3ζ-ζ associated T cell receptors in mature T cells. Perhaps programmed cell death of thymocytes resulting from TCR cross-linking is mediated by CD3η. If this is the case, CD3η will have an important role in thymocyte selection.

Organization of the Mouse CD3η Gene

The organization of the mouse CD3η gene has been characterized and shown that it is part of one

gene locus that also encodes CD3ζ on chromosome 1. The NH_2-terminal sequence of CD3ζ and CD3η, which share the same leader peptide and are identical through amino acid 122 of each mature protein, are encoded by exons 1-7. However, exons 8 and 9 are differentially spliced to give rise to CD3ζ and CD3η: exons 1-8 encode CD3ζ and exons 1-7 plus 9 encode CD3η. RNase protection analysis using a probe derived from the junction of CD3ζ and CD3η divergence and RNA from a variety of fetal, neonatal and adult cell types indicates that expression of both gene products is T lineage restricted. Importantly, both CD3ζ and CD3η mRNA expression appears after day 14 and before or on day 16 of fetal gestation. Expression is apparently coordinate since no cell types tested express CD3ζ and CD3η alone. The level of steady-state CD3ζ mRNA is greater than or equal to 40-60 fold that of CD3η. While in immature $CD4^+CD8^+CD3^{low}$ double positive thymocytes and $CD4^+CD8^+CD3^{high}$ or $CD4^-CD8^+CD3^{high}$ single positive thymocytes the respective steady-state CD3ζ and CD3η mRNA levels are equivalent, the amount of receptor-associated CD3ζ and CD3η proteins in double positive thymocytes is ~10 fold less than in single positive thymocytes. Nevertheless, the ratio of CD3ζ to CD3η proteins remains constant in all populations (40-60:1). Furthermore, discordance between mRNA and protein levels for CD3ζ and CD3η is also observed in splenic T cells. Thus, post-transcriptional as well as transcriptional regulatory mechanisms control CD3ζ and CD3η expression during T cell development.

Differential Signal Transduction Via TCR Isoforms

To compare signalling functions of TCR isoforms, MA5.8, a CD3$\zeta^-\eta^-$ variant of cytochrome c specific I-E^k restricted T cell hybridoma 2B4.11, was stably transfected with cDNAs encoding CD3ζ, CD3η or both and resulting clones characterized. The findings indicate that signals inducing Ca^{2+} mobilization, PI turnover and IL-2 production are transmitted by each of the above TCR isoforms. In contrast, tyrosine phosphorylation of the CD3ζ subunit but not the CD3η subunit follows TCR stimulation. Furthermore, the CD3ζ subunit in $CD3\zeta_2$ homodimers but not in CD3ζ-η heterodimers is detectably phophorylated on tyrosine residues. Given the general importance of tyrosine phosphorylation for receptor signalling, it is likely that this difference between TCR isoforms plays a regulatory role in T lineage function by qualitatively or quantitatively altering signalling events.

To directly determine and compare the role of CD3ζ/CD3η structural isoforms in TCR signal transduction pathways, signalling function of four different types of TCR isoforms, derived from transfection of MA5.8, a CD3ζ-η variant of the cytochrome c specific I-E^k restricted T cell hybridoma 2B4.11 were characterized (Sussman, J.J. et al., Cell 52:85-95 (1988)). The transfections expressed $CD3\zeta_2$, $CD3\eta_2$, $CD3\zeta_2$/CD3ζ-η or $CD3\zeta_2$/CD3ζ-η/$CD3\eta_2$ in association with the pentamer Tiα-βCD3$\gamma\delta\epsilon$ TCR complex.

Diagnostic and Therapeutic Applications

The invention can be used for detecting CD3η in T lymphocyte lineage cells. According to the invention, DNA is obtained from the genome of the cells to be assayed and is fragmented into a multiplicity of DNA fragments. The resulting DNA fragments are hybridized with a probe containing nucleic acid sequences complementary to DNA encoding CD3η surface receptor subunit protein (e.g., all or a portion of the DNA sequence of Figure 1 or a functional equivalent thereof). The DNA is hybridized with the probe under conditions which allow the probe to bind to the DNA fragments having DNA sequences complementary with the gene. DNA fragments which are bound to the probe are detected.

DNA used as a probe can be obtained as described herein (e.g., using standard cloning techniques), can be obtained from cells in which they occur naturally, or can be synthesized chemically or mechanically, using known techniques. The DNA used as a probe can have all or a portion of the DNA sequence of Figure 1 or a functional equivalent of the DNA of Figure 1.

Alternatively, antibodies against the encoded CD3η protein can be produced using known techniques, including anti-heterosera production. Monoclonal antibodies can be produced employing cells which produce antibodies to the CD3η protein by utilizing typical fusion techniques for forming hybridoma cells. Basically, these techniques involve the fusing of the antibody-producing cell with a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and is capable of producing the desired antibody. The hybrid cells are then cultured

under conditions conducive to the production of antibody after which antibody is collected from the cell culture medium. Such techniques for producing monoclonal antibodies have been well described in the literature. See, for example, U.S. Patent Nos. 4,172,124 and 4,196,265, issued to Hilary Koprowski et al., the teachings of which are hereby incorporated by reference.

CD3η present in tissue can also be detected by immunochemical assay. A sample of tissue to be assayed is incubated with antibodies against CD3η. Complexes formed between the antibody and the tissue sample are detected and can be quantitated, and is indicative of the presence, absence, or inappropriate expression/processing of CD3η protein in the tissue.

The transformed cells which express the CD3η surface receptor subunit can be used to screen for substances which are agonistic or antagonistic to CD3-mediated cellular response, such as drugs, chemicals and receptor-specific antibodies. In a screening method for agonistic substances, the transformed cell is contacted with a substance to be tested under conditions (including physiological levels of calcium) which would permit the substance to complex with the CD3η surface receptor. Stimulation of the CD3η receptor is determined by a change in inositol phosphate concentration, whereby an increase is indicative of cell activation. Alternatively, a change in intracellular calcium concentration can also be detected since an increase in calcium is also a consequence of receptor stimulation and inositol phosphate production. Thus,

inositol phosphate and/or calcium concentration are measured within the cell before and after the cell is contacted with the substance.

This system can be used to screen for substances which can block the CD3η surface receptor and inhibit CD3-mediated cellular response. The transformed cell is contacted with a substance to be tested for blocking activity and with a CD3-activating agent (i.e., a compound known to mediate CD3 activation of the cell) under conditions which would permit CD3 stimulation by the activating agent. Inositol phosphate and/or intracellular calcium concentration are then measured as indicative of blocking activity of the substance.

The cytoplasmic domain of CD3η T lymphocyte receptor subunit can be exploited to provide screening assays for substances that interact with cell surface receptors other than CD3. The CD3η cytoplasmic domain can be linked to a non-CD3 extracellular domain to make a chimeric receptor. The chimeric receptor has the extracellular region of a receptor of choice, but the intracellular domain of the CD3 receptor which, thus, produces a CD3-type intracellular. The chimeric receptor is made by producing an expressible chimeric gene construct which can be used to transform an appropriate host cell. The chimeric gene construct comprises DNA encoding the extracellular domain of a non-CD3 cell surface receptor; DNA encoding the transmembrane domain of a cell surface receptor (either CD3η or non-CD3); and DNA encoding the cytoplasmic domain of human CD3η glycoprotein or any portion of the domain which functions for transduction of a CD3-mediated T lymphocyte activation signal into a cell. DNA encoding

the transmembrane domain can correspond to DNA encoding the transmembrane domain of any cell surface receptor.

The invention will be further illustrated by the following examples:

Example 1
Molecular Cloning of the CD3η Subunit

Materials

All restriction endonucleases were from New England Biolabs (Beverly, MA). SP6 and T7 RNA polymerase, RNasin, rNTPs, RQ1 DNase, and wheat germ extract were from Promega Biotec (Madison, WI). G(5')ppp(5')G was from Pharmacia (Uppsala, Sweden). Translation grade ^{35}S-methionine (>1000 Ci/mmol; 1 Ci = 37 GBq) was from NEN Du Pont (Wilmington, DE). Protein molecular weight markers were from BioRad (Richmond, CA). Premixed Acrylamide and Bisacrylamide (Protogel) was from National Diagnostics (Manville, NJ). A full length human CD3ζ cDNA insert in pGEM3Z (referred to here as pGEM3Z-ζ) was provided by A.M. Weissman (Experimental Immunology Branch, NCI, National Institutes of Health, Bethesda, MD). 3DO54.8 was provided by P, Marrack (National Jewish Hospital, Denver, CO). M12.1.4 cells were from L. Glimcher (Harvard School of Publich Health, Boston, MA).

Protein Purification and Microsequencing

To structurally characterize CD3η, anti-CD3ε MAb was used to batch purify TCR subunits and the subunits resolved into individual components by two dimensional (2-D) diagonal gel electrophoresis. The murine OVA-

specific, I-A^d restricted T-T hybridoma cell line, 3DO54.8 (Haskins, K., et al., J. Exp. Med. 157:1149-1169 (1983)); was grown in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin-G 50 μg/ml streptomycin, 50 μM 2-mercapto-ethanol and 1 mM sodium pyruvate in spinner culture. For each 2-D gel electrophoresis, 2 x 10^9 murine 3DO54.8 cells were lysed for 2 h in 20 ml of 1% digitonin lysis buffer (1% digitonin, 150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM PMSF, 10 mM iodoacetamide, 10 μg/ml aprotinin) and the post-nuclear supernatants were prepared by centrifugation at 12,000 G for 30 min. Immunoprecipitation was performed overnight at 4°C with 0.2 ml of Sepharose beads coupled with an anti-murine CD3ε IgG monoclonal antibody (MAb) 145.2C11 (2C11) (3-5 mg/ml). Thereafter, the pelleted beads were washed once by 0.05% digitonin/150 mM NaCl/20 mM Tris, pH 7.4, twice by 150 mM NaCl/20 mM Tris, pH 7.4 and once by 20 mM Tris, pH 7.4. The protein was eluted from beads by boiling for 5 min in 50 μl Laemmli non-reducing sodium dodecasulfate ($NaDodSO_4$) sample buffer. Samples were subjected to electrophoresis in 12.5% polyacrylamide using the buffer system of Laemmli. The gel was then extracted and equilibrated with reducing Laemmli $NaDodSO_4$ sample buffer for 20 min before electrophoresis in a 12.5% reducing $NaDodSo_4$-PAGE. After 2-D diagonal gel electrophoresis, the protein sample was blotted to polyvinylidenefluoride (PVDF) membrane and visualized and quantified by Coomassie blue staining. Approximately 20-50 ng (1-2 picomole) of each CD3ζ-η subunit were isolated from each blot.

Spots corresponding to individual proteins were cut out of 40 PVDF blots and loaded into the Applied Biosystems Model 470A Gas Phase Sequencer (Hewick, R.M. et al., J. Biol. Chem. 256:7990-7997 (1981)) with the 120A PTH Analyzer on line. Each sample was sequenced with the 03RPTH program. After eight cycles, it was apparent that both CD3ζ and CD3η proteins were N-terminally blocked so the 03RPTH run was interrupted after tetrafluoroacetic acid (TFA) cleavage and chlorobutane extraction.

Anti-CD3ϵ MAb (2C11) immunoprecipitates of surface iodinated 3DO54.8 cells lysed in digitonin identify all the known components of the T cell receptor complex. As shown in the autoradiograph of the two dimensional non-reducing, reducing diagonal gel in Figure 5, the Ti α, β heterodimer at 40-60KD as well as CD3 γ, δ and ϵ at 20, 25 and 26KD are readily detected. 2×10^7 3DO54.8 cells were labeled with 1 mCi ^{125}I (Amersham, UK) using lactoperoxidase catalyzed surface radioiodination. Molecular weight markers and their relative mobilities (shown in parentheses) are as follows: 97 (94) KD, phosphorylase B; 68 (68) KD, BSA; 45 (45) KD, OVA; 31 (29) KD, carbonic anhydrase; 22 (20) KD, soybean trypsin inhibitor; and 14 (14) KD, lysozyme.

In addition, two other dimers are identified: a CD3ζ-ζ homodimer (16KD subunit) and a CD3ζ-η heterodimer (16KD and 22KD subunits). Consistent with previous reports, the frequency of incorporation of CD3ζ into the homodimer is at least one order of magnitude greater than its incorporation into the ζ-η heterodimer. Baniyash, M. et al., J. Biol. Chem.

263:9874-9878 (1988). Note that the unlabeled spot at 26KD below the diagonal line represents a CD3ε disulfide-linked homodimer of unknown significance.

CNBr cleavage was subsequently performed (gas phase) in a sealed dish with solid CNBr and 70% TFA in separate vessels. After 5-12 h at room temperature in the dark, residual CNBr/TFA vapors were blown away with argon in the assembled sequencer cartridge and sequencing was resumed with the 03RPTH run. Although Nelson Analytical Software was used for data reduction, the low signal level encountered required visual assessment of chromatograms. Table 1 lists the residues observed (underlined) in individual successive cycles relative to expected residues derived from the CD3ζ cDNA sequence where cleavage should occur after methionine residues at positions 76, 99 and 128 of the mature protein.

TABLE 1

MICROSEQUENCING ANALYSIS OF CNBr FRAGMENTS OF MURINE CD3 ZETA AND ETA PROTEINS DERIVED FROM PREPARATIVE 2-D PVDF BLOTS

Protein	Fragment #	Cycle #	Sequence* 1 5 10 15
Zeta	1	(76)	G G K Q Q R R R N P Q E G V Y N A (92)
	2	(99)	A E A Y S E I G T K G E R (111)
	3	(138)	Q T L A P R (143)
	unassigned residues		
Eta	1		G G K Q Q R R R N P Q E G V Y N A
	2		A E A Y S E I G T K G E R
	3		Q T L A P R
	unassigned residues		P V L G A L

*Residues underlined are those seen that were judged to be significantly above background. Certain residues such as K, R, S and T are not discernable at this low level of sequencing due to poor recovery.

Although the observed signals were all at the level of one picomole, residues in appropriate positions of each of the three corresponding predicted fragments were detected in the empirically derived CD3ζ CNBr fragments. Parallel sequence analysis of an equivalent amount of CD3η identified residues corresponding to known positions of the CD3ζ CNBr fragments 1 and 2. This result unequivocally demonstrates a structural relatedness of CD3ζ and η polypeptides and is consistent with earlier results using anti-peptide heteroantisera which documented crossreactivity of anti-CD3ζ peptide sera with CD3η. Orloff, D.G. et al., J. Biol. Chem. 264:14812-14817 (1989). Perhaps more importantly, in contrast to results obtained with CD3ζ, Table 1 shows that the CD3η sequence lacked any residues corresponding to CD3ζ fragment 3. From the above results, it is concluded that CD3 η and ζ are related proteins but that CD3η structure is distinct from ζ within its carboxy terminal region.

cDNA Library Construction and Screening

To clone CD3η, 274 bp CD3ζ cDNA derived by polymerase chain reaction (PCR) was utilized to screen a murine thymocyte cDNA library and identified 27 positive clones. A mouse thymus cDNA library in the lambda ZAP vector was purchased from Stratagene (La Jolla, CA). The library was made by oligo-dT priming of thymus RNA isolated from 6-8 week old B6/CBA R1J females. The library was screened with a probe isolated by using the PCR to amplify a portion of the murine CD3ζ chain coding region from total thymus RNA. The oligonucleotides used for this reaction were

constructed from published sequences. Weissman, A.M. et al., Science 239:1018-1021 (1988); Baniyash, M. et al., J. Biol. Chem. 264:13252-13257 (1989). The sense oligonucleotide for the PCR was 5' GAGCTCAATCTAGGGCGAAG 3' and is encoded in exon IV. The antisense oligonucleotide was 5' AGCGAGGGGCCAGGGTCTGC 3' and is encoded in exon VIII. RNA from Balb/c murine thymus was reverse transcribed using an oligo-dT primer and reverse transcriptase and subsequently amplified for 40 cycles (Techne machine: denaturation 1 min at 94°C, hybridization 1 min at 55°C, extension 0.8 min at 72°C, final extension 10 min at 72°C) and the resultant 274 base pair product was isolated using a low melting point agarose gel and labeled using the random hexamer priming method. Feinberg, A. and B. Vogelstein, Anal. Biochem. 132:6-13 (1983).

After plaque purification, inserts from each of these clones were hybridized in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 50 mM $NaPO_4$, pH 6.5, 0.1% $NaDodSO_4$, 10 μg/ml denatured salmon sperm DNA at 42°C. Washes were performed at room temperature in 2 x SSC-0.1% $NaDodSO_4$ for 30 min and at 65°C in 1 x SSC-0.1% $NaDodSO_4$ for 60-90 min. Positive phage clones were plaque purified and mini-preps of each phage prepared. DNAs were restricted with EcoRI, electrophoresed and blotted to Zeta probe membranes (Biorad, Richmond, CA). The membranes were separately hybridized in 6 x SCC, 5 x Denhardt's solution, 0.5% $NaDodSO_4$, 10 μg/ml denatured salmon sperm DNA to CD3ζ-derived oligonucleotide 5' AACCCCCAGGAAGGCGTATA-CAAT 3' (bp 415-438) at 64°C and to CD3ζ-derived oligonucleotide 5' CAGACCCTGGCCCCTCGC 3' (bp 577-594)

at 54°C (numbering according Weissman, A.M., et al., Science 239:1018-1021 (1988)). Filters were washed at the hybridization temperature for 20 min in 6x SSC, 0.5% $NaDodSO_4$. All oligonucleotides were synthesized on an Applied Biosystems Model 381A DNA synthesizer.

Inserts from 26 to 27 clones hybridized with both oligonucleotides whereas one clone, termed λzap17, hybridized with oligonucleotide 415-438 but not 577-594. This potential CD3η clone along with the largest CD3ζ inserts were subcloned from the λzap vector using in vivo excision (Stratagene). Plasmid DNA, termed pBS17, was prepared and sequenced on both strands by the method of Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977) using sequence specific oligonucleotides as primers. As shown in Figure 1, the complete nucleotide sequence of the pBS17 insert is 1362 bp long and contains a polyA+ sequence at its 3' end. The positions of the predicted signal peptidase cleavage site (↓), transmembrane region (-), CNBr fragmentation sites (→) and the polyadenylation signal are shown. Note that the last 6 As residues of the insert's polyA+ tail are not shown. The CD3η nucleotide sequence between the arrowheads (bp 35-568) is identical with that of CD3ζ. Baniyash, M. et al., J. Biol. Chem. 264:13252-13257 (1989). An open reading frame of 618 bases (positions 139-756) begins with an ATG methionine codon and is flanked by 138 nucleotides of 5' untranslated sequence and 592 nucleotides of 3' untranslated sequence. A polyadenylation signal (AATAAA) is located at bp 1329-1334 followed by a polyA+ tail beginning at bp 1348.

The experimentally derived residues of the CNBr fragment of CD3η are encoded in the pBS17 insert at nucleotide positions 430-477 and 502-531. All fifteen residues identified by protein sequencing match the cDNA sequence precisely. Furthermore, as expected, each of these sequences is preceded by an ATG codon for methionine. The first 21 predicted amino acids of the open reading frame represent hydrophobic residues and likely encode a leader peptide. Based on the method of von Heijne (von Heijne, G., Nucleic Acids Res. 14:4683-4690 (1986)), the signal peptide cleavage site is located amino-terminal to the Gln residue encoded by nucleotides 202-204. This results in a mature protein of 185 amino acids with a predicted molecular weight of 21,001 and theoretical pI=9.75. This molecular weight is in excellent agreement with results of $NaDodSO_4$-PAGE analysis of CD3η. Within the mature protein, there is a single hydrophobic stretch (residues +10 to +30) likely corresponding to a membrane anchoring segment. This sequence is unusual in the presence of aspartic acid at +15. However, the presence of a single negatively charged amino acid has been noted in each of the other CD3 subunits (Clevers, H. et al., Ann. Rev. Immunol. 6:629-662 (1988)) and presumably forms a critical salt bridge with positively charged residues in the Ti α and β subunits. Thus, the protein encoded by pBS17 is a type I integral membrane protein consisting of a 9 amino acid extracellular segment, a 21 amino acid transmembrane segment and a 155 amino acid cytoplasmic segment. By analogy with CD3ζ-ζ homodimers, the cysteine residue at +11 likely forms the intrachain disulfide bond between CD3η and CD3ζ. The

role of the cysteine residue at +164 is unknown. No N-linked glycosylation sites are present in the predicted CD3η sequence.

RNA Blot Analysis

CD3η mRNA expression in various cell types was examined by RNA blot analysis using the CD3η-specific fragment (bp 633-1362) of pBS17 as a probe. RNAs were prepared from cell lines and tissues using guanidium isothiocyanate and polyA selected on oligo-dT columns (Collaborative Research, Lexington, MA) where indicated. RNA samples were electrophoresed under denaturing conditions in 1% agarose gels containing 7% formaldehyde and 20 mM sodium phosphate (pH 7.0). The RNA was transferred in 20 x SSC to nitrocellulose (Schleicher & Schuell, Keene, NH) or to Biotrans nylon membranes (ICN, Irvine, CA) and the filters hybridized with a CD3η-specific probe (bp 633-1362, pBS17), labeled using the random hexamer priming method (Feinberg, A. and B. Vogelstein, Anal. Biochem. 132:6-13 (1983)) at 1 x 10^6 cpm/ml in 50% formamide/5 x Denhardt's solution/5 x SSC/0.1% $NaDodSO_4$/50 mM $NaPO_4$, pH 7.0/250 μg/ml denatured salmon sperm DNA at 42°C for 12-16 h. Filters were washed at room temperature in 2 x SSC, 0.1% $NaDodSO_4$ for 15-30 min and at 50°C in 0.1 x SSC, 0.1% $NaDodSO_4$ for 30 min, then exposed to film with an intensifying screen for four days for blots containing total RNA and 18 h for blots containing polyA+ RNA (Figure 6).

The 1.7Kb CD3η message was most highly expressed in thymus and the hybridoma 3DO54.8. RNA from spleen gave a low but detectable signal, suggesting that CD3η

is more highly expressed in thymocytes than on mature, splenic T cells. T cell line CTLL gave a positive signal for CD3η while the T cell lymphoma EL4 was negative. CD3η was undetectable in either liver or the B cell lines, A20 and M12. Thus, CD3η appears to be preferentially expressed in T lineage cells where the highest levels are observed in thymocytes.

Synthetic RNA Production and In Vitro Translation

pBS17 was linearized with XbaI and capped, sense RNA synthesized in vitro using T7 RNA polymerase in the presence of G(5')ppp(5')G using Riboprobe Gemini System reagents. pGEM3Z-ζ was linearized with EcoRI and capped sense RNA synthesized with SP6 RNA polymerase. The integrity of the RNA was checked on formaldehyde-MOPS agarose gel electrophoresis. Approximately 1 μg of each RNA was translated in vitro using wheat germ extract. After 2 hr at 25°C, 10 μl of the translation mixture was added to 40 μl of 1x reducing $NaDodSO_4$ sample buffer (10% glycerol, 1% $NaDodSO_4$, 1% 2-methanol, 0.001% Bromophenyl Blue and 62.5 mM Tris pH 6.8) and heated in a boiling water bath for 3 minutes. ^{35}S-methionine labeled samples were then analyzed by $NaDodSO_4$-PAGE using 12.5% acrylamide. Protein bands were visualized by autoradiography using Kodak X-Omat AR film for 12-14 hr at -70°C. Relative molecular weights of the resulting proteins were calculated using the Sizer program from the Ig Suite software package (Intelligenetics, Mountain View, CA) by comparison with known standards.

EXAMPLE 2: Organization of the Mouse CD3η Gene

Antibodies and Cell Lines

Fluorescein isothiocyanate (FITC)-conjugated IgG fraction of goat antiserum against mouse IgG was from Whittaker M.A. Bioproducts (Walkersville, MD). FITC-conjugated IgG fraction of goat antiserum against hamster IgG which does not crossreact with mouse IgG was from Caltag (So. San Francisco, CA). FITC-conjugated anti-Lyt-2 (mouse CD8) monoclonal antibody (MAb) and phycoerythrin (PE)-conjugated anti-L3T4 (mouse CD4) MAb were from Becton Dickinson (Mountain View, CA). IgG fraction of rabbit antiserum directed against mouse IgG was made in our laboratory and purified through protein A-conjugated Sepharose CL-4B. Prestained protein molecular mass markers were from BRL (Bethesda, MD). Rabbit anti-CD3ζ/η peptide antiserum #389 (Orloff, D.G. et al., J. Biol. Chem. 264:14812-14817 (1989)) was generously provided by R.D. Klausner (NIH).

A hamster-mouse somatic B cell hybridoma 145.2C11 (2C11) producing a MAb against mouse CD3ε (Leo, O. et al., Proc. Natl. Acad. Sci. USA 84:1374-1378 (1987)) was generously provided by J.A. Bluestone (NIH). 2C11 MAb was purified from culture supernatant of the hybridoma. A culture supernatant of a mouse B cell hybridoma MKD6 producing a MAb against mouse I-A^d and M12.4.1 cells were generous gifts of L. Glimcher (Harvard School of Public Health, Boston, MA). A culture supernatant of a B cell hybridoma producing a MAbJ11D (Bruce, J. et al., J. Immunol. 127:2496-2503 (1981)) and a culture

supernatant of a B cell hybridoma producing a MAb against mouse CD3ε, 500A2, which was concentrated ten fold were kind gifts from A.M. Kruisbeek (NIH). Cell lines representative of days 14 and 17 fetal mouse thymocytes were derived by transformation with the retroviral construct, J2 (Rapp, U.R. et al., Nature (London) 317:434-438 (1985)), and were also generously provided by A.M. Kruisbeek.

Genomic Library Screening and Restriction Mapping

The 1.9.2 genomic library was constructed from the T-T hybridoma, 1.9.2, by partial Sau3A digestion of 1.9.2 DNA and ligation into the BamHI site of the phage vector λEMBL3. The 1.9.2 library and 1.9.2 DNA were the kind gifts of A. Winoto (Whitehead Inst., Cambridge, MA) (Winoto, A. et al., Nature (London) 324:679-682 (1986)). The Ar5 genomic library constructed by MboI partial digestion of the arsonate-reactive mouse inducer T cell clone Ar5 DNA and ligation into the BamHI site of λCH35 was provided by K.-N. Tan (Dana-Farber Cancer Institute, Boston, MA) (Tan, K.-N. et al., Cell 54:247-261 (1988)). BW5147 DNA was a gift of L. Glimcher (Harvard School of Public Health, Boston, MA); Balb/c, AKR, B10.A(5R) and A/J spleen DNAs were from Jackson Labs (Bar Harbor, ME). B10.BR spleen DNA was prepared as described (Maniatis, T. et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)).

Phage genomic libraries were screened using CD3ζ and CD3η specific probes prepared by digesting pBS23 (CD3ζ) with EcoNI and EcoRI and pBS17 (CD3η)

with AvaII and EcoRI (Jin, Y.-J. et al., Proc. Natl. Acad. Sci. USA 87:3319-3323 (1990)). The probe for isolation of the 5' clones was prepared using a modification of the protocol for rapid amplification of cDNA ends (RACE) (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA 85:8998-9002 (1988)). All probes were labeled by the random priming method (Feinberg, A. and B. Vogelstein, Anal. Biochem. 132:6-13 (1983)). Phages were initially characterized by BamHI, HindIII, PstI and PvuII digestion (all restriction enzymes were from New England Biolabs, Beverly, MA) followed by agarose gel electrophoresis and blotting onto Zeta-Probe membranes according to the manufacturer's recommendations (Biorad, Richmond, CA). Phage blots were hybridized with kinased oligonucleotides specific for CD3ζ exon 1-8 (Baniyash, M. et al., J. Biol. Chem. 264:13252-13257 (1989)) and the unique CD3η sequence (Jin, Y.-J. et al., Proc. Natl. Acad. Sci. USA 87:3319-3323 (1990)). All oligonucleotides were synthesized on an Applied Biosystems model 381A synthesizer.

Restriction mapping of the recombinant phage was performed as described (Rackwitz, H.R. et al., Gene 30:195-200 (1984)) with BamHI and HindIII enzymes. Partial digestion products were selectively labeled at the right cohesive λEMBL3 terminus by hybridization with a ^{32}P-labeled oligonucleotide complementary to the single-strand cohesive end site. After gel electrophoresis and autoradiography, the restriction map was directly determined from the ladder of partial digestion

products and confirmed by analysis of end-labeled products of complete digestion with the same enzymes. Mapping distances are accurate to within 100 base pairs.

Sequence Analysis

The initial characterization of λZE3.20 determined that a 2 Kb PstI fragment hybridized to an oligonucleotide derived from CD3η region 3' to the point of divergence between CD3ζ and CD3η. This PstI fragment was isolated by gel electrophoresis and subcloned into the PstI site of pGEM3zf- (Promega, Madison, WI). Plasmid DNA was sequenced by the dideoxy method (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)) using Sequenase kits (US Biochemical Corp., Cleveland, OH). Oligonucleotide primers for sequencing were derived from the CD3η cDNA sequence and the SP6 and T7 promoter specific primers which lie within the vector.

Analysis of Genomic and Phage DNAs by Southern Blots and Polymerase Chain Reactions (PCR)

For analysis of the genomic DNAs, 10 μg DNA was digested by BamHI or HindIII and electrophoresed on a 0.8% agarose gel. DNA was transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) according to the method of Southern (Southern, E., J. Mol. Biol. 98:503-517 (1975)). Phage DNAs were digested and electrophoresed on the same gel as the genomic DNAs for the comparison of hybridizing fragment sizes. Probes for the genomic Southerns

were random primed labeled (Feinberg, A. and B. Vogelstein, Anal. Biochem. 132:6-13 (1983)) EcoRI inserts of pBS23 (CD3ζ) and pBS17 (CD3η) encoding the entire cDNAs.

For PCR analysis of the distance between exons 4 and 8 in phage and genomic DNAs, a sense oligonucleotide in exon 4 from bp 360-379 (5'-GAGCTCAATCTAGGGCGAAG-3') and an antisense oligonucleotide in exon 8 from bp 633-614 (5'-AGCGAGGGGCCAGGGTCTGC-3') were used. Reactions were according to the manufacturer's instructions using a GeneAmp Kit (Perkin Elmer Cetus, Norwalk, CT). Templates were 200 ng of genomic DNA or 2 μl of a high titer phage stock. Amplification was carried out for 35 cycles on a Techne machine using 1 min denaturation at 94°C, 1 min hybridization at 65°C, 2.5 min extension at 72°C with a final extension of 10 min at 72°C. For determination of the exon 8 length in recombinant phage and genomic DNAs, reactions were carried out as described above using a sense oligonucleotide from exon 8 bp 571-587 (5'-GTCTCAGCACTGCCACC-3') and an antisense oligonucleotide at the 3' end of exon 8 from bp 1673-1654 (5'-GCTACCCCAGGCTTCACCAC-3') for 35 cycles as described above except that the extension cycle time at 72°C was 1.5 min. PCR products were run on agarose gels, blotted to Zeta-Probe membranes and hybridized to the exon 6 specific oligonucleotide bp 501-521 (5'GAAGCCTACAGTGAGATCGGC-3') in the case of the exon 4-8 PCR and above mentioned bp 633-614 exon 8 oligonucleotide for the exon 8 PCR. All oligonucleotides for PCR in this section are

numbered according to the reference (Baniyash, M. et al., J. Biol. Chem. 264:13252-13257 (1989)).

Preparation of Lymphoid Cell Populations

Single cell suspensions of thymocytes and splenocytes were prepared from 4 week old Balb/c mice (Jackson Labs, Bar Harbor, ME or Taconic Farms, Germantown, NY). For splenic T cells, 6-7 x 10^8 splenocytes were suspended in 6 ml of HBSS containing 5% (v/v) FCS and mixed with 2 ml of a culture supernatant of MKD6 and 200 μg rabbit IgG directed against mouse IgG. To make splenic non-T cells, 3-4 x 10^8 splenocytes were suspended in 5 ml of concentrated 500A2 culture supernatant. After an incubation on ice for 30 min, cells were collected by centrifugation, resuspended in 25% (v/v) rabbit complement (Pel-Freez Biologicals, Rogers, AR) in HBSS containing 5% FCS and further incubated at 37°C for 30 min with continuous rotation. Dead cells were then removed by Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) density centrifugation. After 2 cycles of the above procedure, approximately 1.5 x 10^8 cells were recovered from both populations. For $CD4^+CD8^+CD3^{low}$ double positive thymocytes, 5 x 10^8 thymocytes were suspended in 3 ml of concentrated 500A2 culture supernatant. For $CD3^{high}$ single positive thymocytes, 1.5 x 10^9 thymocytes were suspended in 7 ml of J11D culture supernatant as described (Crispe, I.N. et al., J. Immunol. 139:3585-3589 (1987)). After an incubation on ice for 30 min, cells were collected, resuspended in 10% (v/v) rabbit

complement (Cedarlane Laboratories, Hornby, Ontario, Canada) in HBSS containing 5% (v/v) FCS and further incubated at 37°C for 30 min with continuous rotation. Dead cells were removed as described above. Approximately 1.5 x 10^8 cells were recovered for both populations. Phenotypes of those cells were examined by flow cytofluorometry with a FACScan (Beckton Dickinson, Mountain View, CA). In general, purity of each cell population was approximately 90% as assayed by TCR expression. Double positive populations contained at most 10-20% double negative cells. Fetal thymuses were obtained from timed pregnant C57BL/6 mice at the National Institutes of Health (day 1 = day at which vaginal plugs were first observed) and kindly provided by A.M. Kruisbeek.

RNA Preparation and Analysis

pBSΔ17 was derived from the mouse CD3η cDNA clone pBS17 as follows: a portion of the CD3η cDNA, the StyI/SmaI fragment, containing the 3' 681 bp of exon 9 was excised from pBS17. The StyI 5' overhang was filled with Klenow and the linearized plasmid was ligated to obtain pBSΔ17. Antisense RNA was synthesized from pBSΔ17 linearized with AccI using T7 polymerase, Riboprobe Gemini System kits (Promega Biotech, Madison, WI) and 100 μCi of ^{32}P-UTP according to the previously described method (Melton, D.A. et al., Nucleic Acid Res. 12:7035-7056 (1984)). Antisense RNA probe (5 x 10^5 cpm) and total cell RNA were hybridized in 30 μl of a solution consisting of 80% formamide, 0.4 M NaCl, 1

mM EDTA, 40 mM PIPES, pH 6.7 at 45°C. After 18 hrs the RNAs were digested for 1 hr at 30°C by adding 300 µl of a solution consisting of 5 mM EDTA, 0.3 M NaCl, 10 mM Tris/HCl, pH 7.5 and 4 µg/ml RNase T1 or 2 µg/ml RNase T1 + 10 µg/ml RNase A. After proteinase K digestion, phenol/chloroform:isoamyl-alcohol extraction and ethanol precipitation, half of each sample was analyzed on a 5% denaturing polyacrylamide gel. RNA for analysis was prepared from all cells and tissues by the guanidium thiocyanate method (Chirgwin, J.M et al., Biochemistry 18:5294-5299 (1980)).

Immunoprecipitation and Western Blot Analysis

Portions of the thymocyte and splenic subpopulations prepared for RNA analysis as described above were obtained for simultaneous protein analysis. After thrice washing with TBS (25 mM Tris pH 7.5, 0.8% NaCl, 0.02% KCl), cells were lysed at 2 x 10^7 cells/ml in 1% digitonin lysis buffer [1% digitonin (Sigma) in TBS containing iodoacetamide (10 mM), leupeptin (10 µg/ml), antipain (50 µg/ml), PMSF (1 mM), soybean trypsin inhibitor (10 µg/ml), aprotinin (10 µg/ml), and pepstatin (1 µg/ml) (all from Sigma)] by rotating at 4°C for 4 hr. Following centrifugation for 20 minutes at 3500 rpm, the soluble fraction from 2 x 10^8 cells was immunoprecipitated by rotating for 2 hours at 4°C with 50 µl 2C11-coupled Sepharose CL-4B (4-5 mg/ml bed volume). Antigen-antibody-bead complexes were removed by centrifugation at 1100 rpm for 5 min. The depleted supernatant was subjected

to a second round of immunoprecipitation with 2C11-Sepharose beads as described above, or in some instances, with 25 μl Rabbit anti-CD3ζ/η peptide antiserum #389 and 50 μl Protein A Sepharose beads. Antigen-antibody-bead complexes were washed sequentially with 4 ml lysis buffer, 7 ml 0.05% digitonin/TBS/1mM PMSF, 5 ml TBS, and 1 ml 10 mM Tris pH 7.5. Antigen-antibody complexes were solubilized in 100 μl non-reducing Laemlli sample buffer at 100°C for 3 minutes. Aliquots containing 2×10^8 cell equivalents or two-fold serial dilutions of an index sample were resolved by one-dimensional non-reducing SDS-PAGE using 12.5% acrylamide and transferred to nitrocellulose (Clayton, L.K. et al., J. Exp. Med. 172:1243-1253 (1990)). Blots were probed with a 1:200 dilution of rabbit anti-CD3ζ/η peptide antiserum #389 followed by ^{125}I-Protein A (0.2 μCi/ml) as described (Orloff, D.G. et al., J. Biol. Chem. 264:14812-14817 (1989)). Autoradiography was at -70°C for 4 days on Kodak X-Omat AR X-ray film. Molecular weight standards used were bovine serum albumin (71kDa), ovalbumin (45kDa), carbonic anhydrase (28kDa) and β-lactoglobulin (18kDa).

RESULTS AND DISCUSSION

Isolation and restriction analysis of recombinant phage containing the CD3η gene segments

Genomic libraries from the mouse T-T hybridoma 1.9.2 and the Ar5 mouse T cell clone were screened using CD3ζ and CD3η-specific cDNA probes. Three

overlapping phages were isolated from the former and eight from the latter. The restriction maps of the three phages derived from the 1.9.2 library are shown in Fig. 7. Restriction analysis using BamHI and HindIII and Southern blotting with exon-specific oligonucleotide probes demonstrated that each phage contains CD3ζ exons 2-8 (Baniyash et al., J. Biol. Chem. 264:13252-13257(1989)). In addition, phage λZE3.2 and λZE3.20 contain a CD3η-specific hybridizing fragment. Because CD3ζ exon 1 is known to be located >20Kb upstream of CD3ζ exon 2 (Baniyash et al., J. Biol. Chem. 264:13252-13257 (1989)), it is not surprising that all three phages lack exon 1. Two genomic clones containing the 5' sequence of CD3ζ including exon 1 were subsequently isolated from the 1.9.2 genomic library using a RACE-derived probe. These 5' clones did not hybridize to an oligonucleotide probe corresponding to the 5' untranslated region (UTR) CD3η-specific 34 base pairs noted previously (Jin et al., Proc. Natl. Acad. Sci. USA 87:3319-3323(1990)), suggesting that the 5' UTR CD3η sequence is not present in these clones or that it may be disrupted in the genome by one or more introns. As indicated by Fig. 7 (dashed lines), the sizes of the BamHI and HindIII fragments containing exon 8 were different in each phage analyzed (see below). Collectively, the physical maps of these phages (Fig. 7) are consistent with a previous report describing the intron-exon structure of the CD3ζgene (Baniyash et al., J. Biol. Chem. 264:13252-13257(1989)). More importantly, the data herein show that the CD3η gene is part of the same genetic region as CD3ζ.

Analysis of CD3η-specific coding and 3' untranslated gene segment

Digestion of λZE3.20 with PstI followed by Southern blotting using an exon 9 specific oligonucleotide identified a convenient 2Kb PstI fragment which was subcloned into pGEM3zf- and sequenced using CD3η-specific oligonucleotides as primers. Sequence analysis revealed that the CD3η-specific portion (aa 123 to 185) is encoded by a single exon (Fig. 8). The genomic sequence is identical to that found in the CD3η cDNA (Jin et al., Proc. Natl. Acad. Sci. USA 87:3319-3323(1990)) and consists of 782 bp encoding the unique portion of the CD3η protein as well as the entire 3' UTR. A canonical splice acceptor site is present at the 5' end of this exon (Breathnach et al. Ann. Rev. Biochem. 50:349-383(1981)).

Given the NH_2-terminal identity and COOH-terminal divergence of CD3ζ and CD3η sequences, it was previously suggested that CD3η might be an alternatively spliced form of a gene encoding both CD3ζ and CD3η (Jin et al. Proc. Natl. Acad. Sci. USA 87:3319-3323(1990)). The point of divergence between the CD3ζ and CD3η cDNAs occurs at CD3η bp 568 (numbering according to Jin et al. supra (1990)), 1 bp beyond the splice junction at bp 567. However, this apparent discrepancy results from the fact that both exon 8 (Baniyash et al. J. Biol. Chem. 264:13252-13257(1989)) and the CD3η specific exon contain a guanine nucleotide as the first base. This exon was termed exon 9 to reflect the fact that

CD3ζ and CD3η are part of the same genetic locus. Thus as predicted, CD3ζ and CD3η are products of alternatively spliced transcripts arising from a common locus as schematically depicted in Fig. 7.

Analysis of genomic DNAs and recombinant phage by Southern blots and PCR

Genomic Southern analysis of BamHI and HindIII restricted DNAs derived from the 1.9.2 and BW5147 cells as well as splenocytes from Balb/c, A/J, B10.A(5R), AKR and B10.BR was performed using the entire EcoRI inserts of pBS23(CD3ζ) and pBS17(CD3η)(Jin et al. Proc. Natl. Acad. Sci. USA 87:3319-3323(1990)), encoding exons 1-8 and exons 1-7 plus 9, respectively, as probes. As shown by BamHI Southern analysis of Balb/c, B10.A(5R) and AKR-derived spleen DNA, the restriction pattern among strains was indistinguishable when probed with the CD3ζ cDNA. Likewise, the fragments hybridizing with the CD3η cDNA were the same in all strains tested. Exon 8 is found on an invariant 4.6Kb fragment in all mouse genomic DNAs. While the recombinant phage DNAs from genomic clones λZE3.20, λZE3.12 and λZE3.2 contain the 3.6Kb and 2.3Kb CD3ζ hybridizing bands found in the cellular DNA, they lack the 4.6Kb BamHI fragment carrying exon 8. Instead, they have a variably sized fragment (3.3Kb to 4Kb) which is distinct in each clone.

To more precisely define the basis for the size difference among these phages with respect to exon 8, PCR was performed on genomic phage clones and

genomic DNAs using amplimers located in exons 4 (sense) and 8 (anti-sense) and on exon 8. The expected fragments of 3.9Kb for the exon 4-8 product and 1.1Kb for the exon 8 product, both of which hybridized to appropriate internal oligonucleotide probes, were obtained on each of five genomic phages examined including λZE3.2, λZE3.12 and λ3.20, two clones from the Ar5 library, as well as from cellular genomic DNAs of A/J, Balb/c, B10.BR splenocytes and 1.9.2 hybridoma cells. These results show that the intronic region 3' to exon 8 and 5' to the first HindIII site between exon 8 and 9 must be the site of variability (see Fig. 7, dashed line). Given the identity of the CD3ζη locus as defined by Southern analysis of cellular DNA and the variability in size of the portion of the intron between exon 8 and 9 noted in mapping the λEMBL3 genomic clones (Fig. 7, dashed line), it appears that the latter region of DNA is altered upon passage in E. coli during the recombinant phage cloning process. Based on the above analysis, it is concluded that the actual size of the exon 8 containing BamHI fragment is 4.6Kb. Thus, exon 8 and 9 are separated by ~5.2Kb within the CD3ζη locus.

The CD3ζη locus and chromosome 1

The identity in CD3ζ and CD3η hybridization patterns detected in Southern analysis of DNAs from all mouse strains examined is not surprising in view of the fact that the CD3ζη locus is found within a conserved region of chromosome 1 in mouse and man.

This region of the chromosome contains a 6000Kb segment which is highly conserved between human chromosome 1 at 1q21-32 and distal mouse chromosome 1 (reviewed in Moseley, W.S. & Selden, M.F. Genomics 5:899-905 (1989)). CD3ζ has been mapped to mouse chromosome 1 (Baniyash et al. J. Biol. Chem. 264:13252-13257(1989)) within this conserved region which also includes complement component 4 binding protein, leukocyte common antigen (CD45), the FcγRII IgG receptor (CDw32) and members of the selectin family of leukocyte adhesion molecules (GMP-140, LHR, ELAM-1 and Ly-22). In addition, this region encodes CD21 (CR2), CD35(CR1), CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), CD1 and CD62. The recessive mutation for generalized lymphadenopathy disease (gld) causing an autoimmune syndrome in C3H/HeJ mice also maps to mouse chromosome 1 (reviewed in Moseley, W.S. & Seldin, M.F. Genomics 5:899-905 1989; Siegelman, M.H., et al. Cell 61:611-622 (1990); and, Knapp, E. et al. (eds) Leukocyte Typing IV, Oxford: Oxford University Press (1990).

The γchain of the high affinity IgE receptor (FcεRIγ) is located on mouse chromosome 1 (Huppi, K. et al. J. Immunol. 143:3787-3791 1989)). This is of particular interest since a high degree of homology has been noted between the FcεRIγ and CD3ζ(Kuster et al. J. Biol. Chem. 265:6448-6452(1990)). Given the relatedness of CD3ζ and CD3η, this similarity in structure can be extended to CD3η and FcεRIγ. The organization of FcεRIγ and CD3ζη exons are analogous in several respects. First, in each gene, exons 1 and 2

contain the leader peptide, the extracellular domain, transmembrane region and first three residues of the cytoplasmic domain. Second, the respective cytoplasmic domains are encoded by exons 21-81 bp in length. The CD3ζ and CD3η subunits are encoded by three additional cytoplasmic domain exons not found in FcϵRIγ. Third, the last exon of each gene contains sequence encoding the carboxy-terminal amino acids of the cytoplasmic domain plus the entire 3' UTR. Finally, significant amino acid homology exists between the region encoded by exon 2 of FcϵRIγ and exon 2 of CD3$\zeta\eta$, and between exon 5 of FcϵRIγ and exon 8 of CD3ζ. In contrast, no carboxy-terminal amino acid homology is apparent between FcϵRIγ and CD3η. The similarities in exon-intron structure, sequence homologies and chromosomal localization of FcϵRIγ and CD3$\zeta\eta$ suggest that gene duplication followed by insertion or deletion of three exons could explain the origins of these two genetic loci.

Analysis of CD3ζ and CD3η mRNA expression

Prior studies demonstrated that thymocytes and peripheral T cells respond differently after T cell receptor crosslinking; the majority of thymocytes do not proliferate and, in fact, die whereas T lymphocytes are triggered to proliferate (Ramarli et al. Proc. Natl. Acad. Sci. USA 84:8598-8602 (1987) and Smith et al. Nature 337:181-184(1988)). Given the aforementioned structural differences in CD3ζ and CD3η proteins, it is possible that differential

CD3η expression during thymocyte development is responsible for these signalling differences. To investigate this possibility through analysis of steady-state mRNA expression, an antisense RNA probe was produced to simultaneously detect and distinguish CD3ζ and CD3η mRNA in RNase protection assays. This probe includes the junction between exon 7 and 9 in CD3η clone pBSΔ17 such that hybridization of CD3η mRNA results in a 173 bp protected fragment whereas CD3ζ mRNA protects a 100 bp fragment following RNase digestion (Figure 9).

RNAs isolated from total adult and fetal thymus, thymocytes and thymocyte subpopulations, v-raf/v-myc retroviral transformed mouse fetal thymocyte lines, splenocytes and splenocyte subpopulations, liver and brain were analyzed for CD3ζ and CD3η expression. Two major protected fragments were observed at 173 and 100 bases by RNAs from T lineage cells from Balb/c (total RNA extracted from $8x10^7$ or $4x10^7$ adult thymocytes and total RNA extracted from $3.75x10^7$ adult thymocytes, double positive TCR^{low} thymocytes, single positive TCR^{high} thymocytes, total splenocytes and splenic T cells) and C57 BL/6 (15μg of total RNA from newborn thymuses, day 18 and day 16 fetal thymuses) animals. In contrast, no protected fragments are observed when the ^{32}P labeled RNA probe was hybridized with Balb/c liver or Balb/c brain RNA or splenic non-T cell RNA, demonstrating that both CD3ζ and CD3η mRNAs are T lineage restricted. Consistent with this notion is the observation that Balb/c kidney, B cell lines A20 and M12.4.1 and the mast cell lines

C1.MC/57.1 (Young et al. Proc. Natl. Acad. Sci. USA 84:9175-9179 (1987)) and IC2 (Koyasu et al. J. Immunol. 134:3130-3136(1985)) also lacked CD3ζ or CD3η mRNA as judged by this protection analysis. A band at 225 bases represented the undigested probe. The presence of excess probe was confirmed by the two-fold increase in hybridization signal generated after doubling the cell equivalent RNA amounts in $8x10^7$ or $4x10^7$ adult thymocytes.

To determine whether there were differences in CD3ζ and CD3η mRNA expression among different subpopulations of T lineage cells, equal numbers of total thymocytes, $CD4^+CD8^+CD3^{low}$ double positive thymocytes and $CD4^+CD8^-CD3^{high}$ plus $CD4^-CD8^+CD3^{high}$ single positive thymocytes as well as total splenocytes and splenic T lymphocytes were isolated from 4 week old Balb/c animals. All populations expressed both CD3ζ and CD3η mRNA (total RNA extracted from $3.75x10^7$ adult thymocytes, double positive TCR^{low} thymocytes, single positive TCR^{high} thymocytes, total splenocytes and splenic T cells). Importantly, densitometry scanning of individual lanes showed that in each sample, the CD3ζ protected band was approximately 20-30 fold more intense than the CD3η band. Assuming that the intensity of these signals is proportional to the absolute amount of mRNA present and correcting for the ^{32}P content of the CD3η protected fragments, it is concluded that the steady-state level of CD3ζ mRNA is approximately 40-60 fold greater than CD3η mRNA in each of the T lineage cell populations examined. Moreover, the steady state amount of CD3ζ and CD3η mRNA is

equivalent between double positive and single positive thymocytes but much lower in splenic T cells. Subsequently, CD3ζ and CD3η mRNA expression was examined during mouse gestation using RNAs (15μg) obtained from C57BL/6 animals at days 16 and 18 of gestation and newborn animals. Again, all samples contained both protected fragments with similar CD3ζ/CD3η mRNA ratios. Similar results were obtained with RNA from thymuses of C57BL/6 mice at 4, 8, 12, 28 and 42 days after birth.

To examine CD3ζ and CD3η expression at a very early stage of thymocyte development, v-raf/v-myc retroviral transformants of day 14 fetal thymocytes were characterized. Two of the five representative clones, termed 35.1 and 32.1 ($CD2^{+}Thy\text{-}1^{low}PgP\text{-}1^{high}CD3^{-}CD4^{-}CD8^{-}$) do not express CD3ζ or CD3η. This result suggests that the CD3ζη locus is not transcriptionally activated until after this stage of gestation. Consistent with this possibility is the finding that similarly derived thymocyte transformants (C3H.B4 and C3H.B1)($CD2^{+}Thy\text{-}1^{low}PgP\text{-}1^{high}CD3^{-}CD4^{-}CD8^{-}$) from day 17 thymocytes express both CD3ζ and CD3η. Assuming these cells are representative of normal thymocytes and in view of the results obtained with the day 16 fetal thymocytes transcription of the CD3ζη gene must begin on gestational days 15 or 16. Although the ratio of CD3ζ to CD3η mRNA in these day 17 transformed clones (10-20:1) is lower than in normal T lymphocytes and thymocytes, the significance of this finding remains to be determined in view of the above results.

Analysis of CD3ζ and CD3η protein expression

To investigate how the above described quantitative differences in CD3ζ and CD3η mRNA level among T lineage subpopulations affected protein expression, $CD3^{low}$ double positive thymocytes, $CD3^{high}$ single positive thymocytes, total thymocytes, and splenic T cells were analyzed for CD3ζ and CD3η protein expression by immunoprecipitation of the TCRs with anti-CD3εMAb and subsequent western blot analysis using an anti-CD3ζ/η heteroantiserum. The levels of both proteins increase during T cell development. Thus, the amount of CD3ζ and CD3η proteins is lowest in $CD4^{+}CD8^{+}CD3^{low}$ double positive thymocytes; and highest in $CD3^{high}$ single positive thymocytes and splenic T cells. As expected, the total population of thymocytes displays an amount of CD3ζ and CD3η protein intermediate between $CD3^{low}$ double positive thymocytes and $CD3^{high}$ single positive thymocytes. That the CD3ζ and CD3η proteins precipitated with the TCR represent the vast majority of these two proteins in the cell was demonstrated by sequential immunoprecipitation with anti-CD3ε followed by rabbit anti-CD3ζ/η antiserum.

To more precisely compare the ratio of CD3ζ protein to CD3η protein between single positive and double positive thymocyte subpopulations, the immunoprecipitate from the single positive thymocytes was serially diluted (1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125×10^{8} cells) immediately prior to SDS-PAGE and western blot analysis. As is

apparent by comparing the signals in the double positive thymocytes ($1x10^8$ cells) with the signals in dilutions of single positive thymocytes containing an equivalent amount of CD3ζ (0.25 and $0.125x10^8$ cells), the amount of CD3ζ in single positive cells is ~8 times that in double positive cells. Furthermore, the ratio of CD3ζ protein to CD3η protein is essentially equivalent in both cell types and is approximately 40-60:1. The ratio of CD3ζ protein to CD3η protein in single positive thymocytes is also identical to that of splenic T cells. Shorter exposure of these blots confirm these conclusions. Collectively these results show that, although the absolute amount of both CD3ζ and CD3η protein increases with T cell maturation, their ratio remains constant during the stages of T cell development examined.

Whereas the amount of CD3ζ and CD3η mRNA found in $CD3^{low}$ double positive thymocytes and $CD3^{high}$ single positive thymocytes is equivalent, this similarity in mRNA expression is not reflected at the protein level: the level of CD3ζ and CD3η protein is lower by an order of magnitude in $CD3^{low}$ double positive thymocytes than $CD3^{high}$ single positive thymocytes. This discordance is further evident in the splenic T cell population, which expresses lower amounts of CD3ζ and CD3η mRNA than $CD3^{low}$ double positive thymocytes but contains substantially more of the corresponding proteins. The observation that CD3ζ and CD3η mRNA levels decrease during T cell maturation while CD3ζ and CD3η protein levels simultaneously increase implies

that CD3ζ and CD3η expression is subject to post-transcriptional regulation. Furthermore, the stable ratio of CD3ζ to CD3η during maturation implies that such post-transcriptional regulation not only controls the absolute level of CD3ζ and CD3η protein, but also maintains stringent control of the ratio of CD3ζ to CD3η protein in the face of changing mRNA levels. These data show that CD3ζ and CD3η expression is transcriptionally and post-transcriptionally regulated during discrete stages of thymocyte development.

CD3ζ and CD3η proteins play important roles in controlling TCR surface expression. It has been shown, for example, that CD3ζ protein salvages Tiα-βCD3γδε pentamer from the lysozomal degradation pathway and routes the TCR to the cell surface (Chen et al. J. Cell Biol. 107:2149-2161(1988)). Data have been generated to suggest that CD3η protein is also capable of functioning in an analogous fashion but results in a TCR surface level lower than that induced by CD3ζ (Clayton et al. J. Exp. Med. 172:1243-1253(1990)). Thus, differential expression of CD3ζ and/or CD3η proteins in different T cell subsets may control the surface level of TCR. The finding of post-transcriptional regulation of CD3ζ and CD3η proteins in double positive TCR^{low} and single positive TCR^{high} thymocytes is consistent with this view. Moreover, the levels of CD3ζ and CD3η protein in these subpopulations correspond to the known differences in cell surface TCR copy number in such subpopulations. Thus CD3ζ and CD3η protein levels may be responsible in part or in

total for the observed surface TCR copy number difference. Presumably the lower TCR expression in immature double positive thymocytes necessitates a higher avidity ligand interaction to activate thymocytes than would be required to activate thymocytes expressing high TCR amounts and as such may dictate a threshold for selection.

It has been proposed that CD3η is involved in signal transduction for negative selection in the thymus (Mercep et al. Science 246:1162-1165(1989)). If so, then one might have expected to find differential expression of CD3ζ and CD3η during thymic differentiation. Given that CD3ζ and CD3η mRNAs and proteins are expressed in T lineage populations in both thymus and spleen, the similarities in ratio of CD3ζ:CD3η mRNAs in all T cell populations examined (fetal, newborn and adult mouse thymocytes and/or double positive and single positive thymocyte subsets) and the increase in steady state CD3η protein levels during T lineage maturation, this proposal seems less likely. The possibility that CD3η may be preferentially expressed in a numerically minor subset of double positive or single positive thymocytes undergoing selection processes cannot be ruled out. In this regard development of CD3ζ and CD3η in thymic development will likely result from studies utilizing transgenic strains of mice expressing purposefully altered levels of CD3ζ and CD3η and strains made devoid of CD3ζ or CD3η by targeted gene destruction.

EXAMPLE 3: Differential Signal Transduction via TCR Isoforms

Cells and Antibodies

Cytochrome c specific I-E^k restricted mouse T cell hybridoma 2B4.11, its variants MA5.8 (Sussman, et al. Cell 52:82-95 (1988)) and the B cell hybridoma LK35.2 were generous gifts of J. Ashwell (NIH, Bethesa, MD). The cells were maintained in RPMI 1640, 10% FCS. MAζ-η301 and MAζ201 were continuously grown in RPMI 1640 containing 10% FCS, 500 μg/ml G418 (Geneticin, Gibco), 0.5μg/ml mycophenolic acid (Sigma), 15μg/ml hypoxanthine (Sigma) and 250μg/ml xanthine (Sigma). The hamster-mouse somatic B cell hybridoma 145.2C11 producing the monoclonal antibody (MAb) 2C11 against mouse CD3ε was generously provided by J.A. Bluestone (NIH) (Leo, O. et al., Proc. Natl. Acad. Sci. USA 84:1374-1378 (1987)). MAb A2B4.2 specific for the Tiα chain of the 2B4.11 TCR was the generous gift of J.A. Ashwell (NIH) (Samelson, L.E. et al., Proc. Natl. Acad. Sci. USA 80:6972-6976 (1983)).

Construction of Expression Plasmids and Maintenance of Cell Lines

The insert of pBS17 (mouse CD3η) (Jin, Y-J. et al., Proc. Natl. Acad. Sci. USA 87;3319-3323 (1990)) was excised with EcoRI and blunted with DNA polymerase I (Boehringer Mannheim). The CD3η insert was ligated to pM2gpt (a generous gift of M. Concino, Procept, Inc., Cambridge, MA) which had

been cut with XhoI and blunted. Ligations were transformed into HB101 and positive colonies identified by colony hybridization. Plasmid DNA was isolated from correct orientation construct of pM2gpt-η and opposite orientation construct of pM2gpt-antisense-η by double banding in CsCl or by Qiagen plasmids kits following the manufacturer's recommendation. Plasmid DNA was prepared for transfection by linearization with FspI. All restrictions enzymes were from New England Biolabs. The pM2gpt vector drives insert expression with the adenovirus major late promoter and provides mycophenolic acid resistance (M. Concino, Personal Communications).

RNA Analysis

Total RNA was isolated from 2B3.11, MA5.8 and transfectants using quanidium isothiocyanate (Maniatis, T. et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY(1982)). RNA concentrations were measured spectrophotometrically and 10μg (for CDη) or 20μg (for CD3ζ) of total RNA as indicated were run per lane on a 1% agarose gel containing 2.2 M formaldehyde and 1 x MOPS buffer solution (Maniatis (1982) supra). The RNA was transferred to nitrocellulose in 20 x SSC and the RNA blots hybridized to the CD3ζ or CD3η specific probes prepared by digesting pBS34 (Mercep, M. et al., Science 242:571-574 (1988)) with EcoNI and EcoRI and pBS17 with AvaII and EcoRI, respectively. The 3' fragments of the cDNAs were isolated in low

melting agarose gels and CD3η or CD3ζspecific probes were labeled by the random-priming method (Feinberg, A.P. and B. Vogelstein, Anal. Biochem. 132:6-13 (1983)). Hybridization was in 50% formamide, 5 x SSC, 5 x Denhardt's, 0.1% SDS, 250 μg/ml denatured salmon sperm DNA, 50 mM $NaPO_4$, pH 6.5 at 42°C for 16-20 h with 1 x 10^6 cpm/ml probe. After washing in 2 x SSC, 0.1% SDS for 15-30 min at room temperature and 0.1 x SSC, 0.1% SDS at 50°C for 30 min, the blot was exposed to Kodak X-Omat AR X-ray film at -70°C for the indicated times.

Western Blotting Analysis

Cells were washed 3 times with TBS and cells were solubilized at 2.5 x 10^7/ml in 1% digitonin (Sigma) in TBS (0.8% NaCl, 0.02% KCl, 25 mM Tris/HCL, pH 7.5) containing 10 mM iodoacetamide, 10 μg/ml leupeptin, 50 μg/ml antipain, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 1 μg/ml pepstatin (all from Sigma) by rotating at 4°C for 2 hr. Following centrifugation for 30 minutes in an eppendorf microcentrifuge, the soluble fraction was incubated with either 50 μl of Sepharose CL-4B (Pharmacia LKB) covalently coupled to 2C11 (4 mg/ml) or 25 μl of rabbit anti-CD3ζ/η antibody #387 (kindly provided by R.D. Klausner) in the presence of 50 μl protein A-Sepharose CL-4B rotating overnight at 4°C. The bead-antibody-antigen complexes were pelleted by centrifugation, the supernatant removed and the beads washed sequentially with 4 ml of lysis buffer, 7 ml of TBS containing 0.05% digitonin and 1 mM

PMSF, 5 ml TBS and 1 ml 10 mM Tris/HCL, pH 7.5. Antigen-antibody complexes were solubilized in 50 μl of non-reducing Laemmli's sample buffer at 100°C for 3 minutes (Laemmli, U.K., Nature 227:680-685 (1970)). Aliquots containing 2.5×10^7 cell equivalents were resolved by 1D-non-reducing SDS-PAGE or 2D-non-reducing/reducing SDS-PAGE using 12.5% acrylamide and transferred to nitrocellulose (Bio-Rad) for 1 hr at 100V in a solution consisting of 25 mM Tris, 192 mM Glycine and 20% MeOH. Following a 2 hr room temperature incubation in TBS containing 5% milk and 0.02% sodium azide, blots were incubated overnight at 4°C with antibody #387 diluted 1:200 in TBS containing 2.5% milk, washed three times with TBS containing 2.5% milk and 0.1% Triton X-100 and incubated for 2 hr at room temperature with ^{125}I-labeled Protein A (New England Nuclear) at 0.2 μCi/ml in TBS containing 2.5% milk. After extensive washing with TBS containing 2.5% milk and 0.1% Triton X-100, blots were exposed for 24 hours at -70°C to Kodak X-Omat AR X-ray film. Migration position of prestained molecular weight markers (BRL) are indicated: 106KD, phosphorylase B; 71KD, BSA; 44KD, ovalbumin; 28KD, carbonic anhydrase; 18KD, β-lactoglobulin; 15KD, lysozyme.

Calcium Mobilization in the Transfectants

Cytosolic Ca^{2+} concentrations were determined as described (Alcover, A. et al., EMBO J. 7:1978-1987 (1988)). 2×10^6 cells were pelleted and resuspended in 200 μl of RPMI 1640 containing 10% FCS and 1 μM of the acetoxymethyl ester of

indo-1 (Molecular Probes). The cells were incubated for 30 min at 37°C and then diluted 10 fold with RPMI 1640 containing 10% FCS. Cells were stimulated with 12 μg 2C11 and 30 μg goat anti-hamster antibody (Caltag). As a control, 10μl calcium ionophore A23187 (2mg/ml, Molecular Probes) was added to MA5.8. Analysis was performed with an Epics V cell sorter.

Measurement of Phosphatidylinositol Hydrolysis

^{3}H-inositol phosphates were separated from the specified cell populations and quantified by methods already described (Berridge, M.J., Biochem. J. 212:849-858 (1983); Beaven, M.A. et al., J. Biol. Chem. 259:7137-7142 (1984)). Incorporation of ^{3}H-inositol into phospholipid was achieved by incubating the T cell hybridomas (10^{7} cells/ml) with 40 μCi/ml of ^{3}H-inositol (37 MBq/ml, New England Nuclear) for 3 h at 37°C in HBSS containing 10 mM HEPES and 0.5% gelatin. After addition of 10 volumes of RPMI 1640 containing 10% FCS, the cells were incubated overnight at 37°C. The cells were then washed and resuspended in HBSS containing 20 mM LiCl at 1 x 10^{7}/ml. After 10 min incubation at 37°C, 5 x 10^{6} cells/time point were aliquoted into eppendorf tubes. LK35.2 cells were washed and resuspended at 1 x 10^{7}/ml in RPMI 1640 containing 10% FCS with 50 μg/ml 2C11 or 100 μM cytochrome c peptide (KKANDLIAYLKQATK) (Clayton, L.K. et al., J. Exp. Med. 172:1243-1253 (1990)). After preincubation for 10 min, the labeled cells were added and the tubes incubated at 37°C until the

indicated time points. The cells were then pelleted and the supernatant discarded. The pellet was lysed with 750 µl of a 100:200:2 mixture of $CHCl_3$:MEOH:HCl. Phases were separated by addition of 200 µl $CHCl_3$ and 200 µl H_2O. After centrifugation, the aqueous phase was diluted in 2.3 ml H_2O and stored at -20°C until column separation was performed. The columns were made from 1.5 ml of Dowex 1 x 8-200 (Sigma) in formate form. After loading the sample, the column was washed with 20 ml of 60 mM sodium formate and 5 mM sodium tetraborate. Inositol phosphates (IP_1, IP_2 and IP_3) were then eluted with 5 ml of 1 M ammonium formate and 0.1 M sodium formate. Radioactivity was determined by scintillation counting in Monoflow 4 (National Diagnostics).

Immunoprecipitation of Phosphorylated Proteins

Cells (1 x 10^7) in 1 ml of the RPMI 1640 medium containing 10% FCS were stimulated by crosslinking the TCR with either anti-CD3ε or anti-Ti mAb and species-specific anti-IgG antibodies. After 30 min stimulation, cells were harvested by centrifugation for 20 seconds at 2,000 x g and resuspended in 0.1 ml of a buffer solution consisting of 150 mM NaCl, 1 mM orthovanadate, 1 mM PMSF, 10 mM Tris/HCl, pH 8.0. Subsequently, cells were lysed in 0.4 ml 0.5% Triton X-100, 10 mM EDTA, 10 mM EGTA, 1 mM NaF, 25 mM Tris/HCl, pH 8.0 for 1 h at 4°C. Insoluble materials were removed by centrifugation for 10 min at 14,000 x g and lysates were precleared for 1 h with 0.1 volume of protein A-Sepharose CL-4B

(Pharmacia) in the presence of 5% FCS. Precleared samples were then immunoprecipitated at 4°C overnight with 0.1 volume of 2C11-coupled Sepharose CL-4B beads (405 mg/ml) or 2 μg of anti-P-Tyr 4G10 (Druker, B.J. et al., N. Eng. J. Med. 321:1383-1391 (1989)) (kindly provided by T. Roberts, Dana-Farber Cancer Institute, Boston) and 0.1 volume of protein A-Sepharose CL-4B. The beads were then washed five times with lysis buffer solution supplemented with 1 mM orthovanadate and 1 mM PMSF and boiled in Laemmli's non-reducing sample buffer solution. Samples were then subjected to 1D-non-reducing SDS-PAGE or 2D-non-reducing/reducing SDS-PAGE (12.5% acrylamide). Proteins were then transferred to nitrocellulose and blotted with the antibodies indicated. Finally, immunoreactive proteins were visualized using alkaline phosphatase-coupled second-step reagents obtained from BioRad. Migration positions of prestained molecular weight markers (BioRad) were: 110KD, phosphorylase B; 84KD, BSA; 47KD, ovalbumin; 33KD, carbonic anhydrase; 24KD, soybean trypsin inhibitor; 16KD, lysozyme.

Determination of IL-2 Activity

For antigenic stimulation, 3×10^5 cells were stimulated in 1 ml RPMI 1640 containing 10% FCS, 50 μM 2-mercaptoethanol and the indicated concentration of cytochrome c peptide in the presence of either $I\text{-}E^k$ expressing B lymphoma LK35.2 cells or B10.BR splenocytes. For CD3 crosslinking in experiment 2 (Table 2), 2×10^4 cells were stimulated in an

Immulon 96 well ELISA plate (Dynatech Laboratories) coated with the indicated amounts of 2C11. After 24 h incubation, supernatants were collected and IL-2 activities were assayed by DNA synthesis of an indicator cell line, CTLL-15G (Gillis, S. et al., J. Immunol. 120:2027-2032 (1978)) (kindly provided by K.A. Smith, Dartmouth Medical School, Dartmouth, NH). Briefly, recombinant IL-2 (Biogen; 4.6 x 10^6 units/mg protein) or samples were serially diluted with the above media in 96 well round bottom microtiter well (75 μl/well). CTLL-15G cells (1 x 10^4 cells in 75 μl) were subsequently added and cultured for 21 h followed by 3h pulse labeling with 1 μCi ^{3}H-thymidine/well. IL-2 activity of each sample was determined in triplicate by comparing each titration curve with that of recombinant IL-2 and presented as units/ml production by 3 x 10^5 cells.

Results and Discussion

MAζ15.4 (expressing CD3ζ_2), MAη61.9 (CD3η_2) and MAζ-η52.9 (CD3ζ_2/CD3ζ-η) were generated by transfection of MA5.8 cells with the expression vector pPink-2 containing the CD3ζ cDNA, CD3η cDNA or both constructs, respectively (Clayton, L.K. et al., J. Exp. Med. 172:1243-1253 (1990)). MAζ201 (CD3ζ_2) and MAζ-η301 (CD3ζ_2/CD3ζ-η/CD3η_2), were generated by transfection of MAζ15.4 cells with the pM2gpt vector containing either the anti-sense (MAζ201) or sense (MAζ-η301) orientation of the CD3η cDNA. MAζ-η301 and MAζ201 express high levels of TCR which are 3-4 fold greater than 2B4.11. This

level is comparable to that of the parental MA15.4 cells. As previously reported (Sussman, J.J. et al., Cell 52:85-95 (1988)), MA5.8 has a more than 5 fold lower surface TCR copy number than 2B4.11. MAζ-η301 expresses CD3η as well as CD3ζ mRNA; the sizes of the transcripts derived from the expression vector are larger than the endogenous 2.0Kb CD3ζ and 1.7Kb CD3η transcripts in 2B4.11. As expected, no transcripts for CD3ζ or CD3η are present in MA5.8.

To analyze TCR-associated CD3ζ and CD3η proteins in these transfectants, the TCR complex was immunoprecipitated from digitonin lysates of MAζ201 and MAζ-η301 with anti CD3ε mAb (2C11), resolved on non-reducing SDS-PAGE and analysed by western blotting with rabbit anti-CD3ζ/η antibody #387 which recognizes both CD3ζ and CD3η (Orloff, D.G. et al., J. Biol. Chem. 264:14812-14817 (1989)). Three types of dimers were observed in MAζ-η301: a $CD3\zeta_2$ homodimer at 32KD, a CD3ζ-η heterodimer at 38KD and a $CD3\eta_2$ homodimer at 42KD. These results unequivocally demonstrate that three different types of CD3ζ/CD3η dimers are associated with the TCR of MAζ-η301 cells. In contrast, MAζ201 expresses only $CD3\zeta_2$. The dimeric nature of the three proteins in MAζ-η301 is shown by 2-D diagonal non-reducing/reducing SDS-PAGE analysis of MAζ-η301 lysates immunoprecipitated and western blotted with antibody #387. The amount of $CD3\eta_2$ detected in the TCR complex was lower than that present in the cells as defined by antibody #387, suggesting that the association of $CD3\eta_2$ to the TCR is weaker than that of $CD3\zeta_2$ or CD3ζ-η. As reported previously,

MAζ-η52.9 cells express CD3ζ_2 and CD3ζ-η but unlike MAζ-η301, do not express CD3η_2 (Clayton, L.K. et al., J. Exp. Med. 172:1243-1253 (1990)). The low level of CD3η in MAζ-η52.9 relative to MAζ-η301 cells seems to account for this difference. Thus, the preference for subunit assembly in a stable TCR complex is likely to be CD3ζ_2>CD3ζ-η>CD3η_2. Of note, transfection of CD3η into MAζ15.4 cells which already express CD3ζ, does not affect the TCR surface level as observed in the resultant transfectant MAζ-η301.

Signal transduction through TCR stimulation was assessed in transfectants as well as 2B4.11 and MA5.8. Crosslinking of the TCR with anti-Ti mAb A2B4.2 results in Ca^{2+} mobilization in all the transfectants and in the parental 2B4.11 cells. In contrast, no Ca^{2+} mobilization was detected in MA5.8. Similar results were obtained when cells were triggered with the anti-Cd3ε mAb. These results indicate that CD3ζ and CD3η either alone or in combination are capable of transmitting signals responsible for Ca^{2+} mobilization. Furthermore, given that inositol 1,4,5-trisphosphate, a product of IP turnover, is implicated as being responsible for a rise in intracellular free calcium (Kuno, M. and P. Gardner, Nature 326:301-304 (1987)), the findings suggested that IP turnover can be stimulated by TCR crosslinking in all transfectants. To directly test this possibility, PI turnover in the same cell populations was measured. Upon stimulation with anti-CD3ε mAb, PI turnover in 2B4.11, MAζ-η301, MAζ201, MAζ15.4, and MAη61.9 as

measured by an increase in total inositol phosphates was readily detected. In contrast, only a low level of PI turnover was observed in these transfectants when the cells were stimulated with 100 μM cytochrome c peptide fragment in the presence of LK35.2 cells as antigen presenting cells (APC). As expected, little, if any, PI turnover was detected in MA5.8. The basis of the disparity between antibody and antigen stimulation is unclear. However, presumably, PI hydrolysis occurs in the transfectants only after substantial TCR aggregation, this is achieved by CD3ε crosslinking but not by antigen/MHC stimulation.

The suggestion of Ashwell and colleagues that PI hydrolysis requires the CD3ζ-η heterodimer was based on the observation that CD3ζ transfectants lacking CD3ζ-η heterodimers underwent little, if any, PI turnover upon antigen/MHC stimulation (Mercep, M. et al., Science 242:571-574 (1988)). However, parallel examination of $CD3\zeta_2$, $CD3\eta_2$ and CD3ζ-η transfectants as shown above reveals low but detectable levels of PI turnover in response to antigen/MHC stimulation in each case. Thus, it is concluded that there is no significant difference in the capacity of CD3ζ and CD3η proteins to transmit signals for PI turnover in MA5.8 transfectants.

To next characterize the ability of various cell types to produce IL-2, 2B4.11, MA5.8 and the transfectants were stimulated with cytochrome c peptide with B10.BR splenocytes as APC and IL-2 secretion was quantitated by measuring proliferation of an IL-2 dependent cell line. The data in Table 2

demonstrate that 2B4.11 and all the transfectants produce IL-2 in an antigen concentration-dependent manner, whereas MA5.8 cells fail to produce IL-2 at any concentration of antigen up to 25 μM. Stimulation of the cells by crosslinking of TCR with immobilized anti-CD3ϵ mAb results in IL-2 production by all the transfectants and MA5.8 cells (Table 2). These results indicate that each of the CD3ζ/CD3η dimers (CD3ζ_2, CD3ζ-η and CD3η_2) can facilitate the transmission of signals responsible for IL-2 production after antigenic stimulation. The fact that MA5.8 cells can produce IL-2 in response to anti-CD3ϵ mAb crosslinking suggests that neither CD3ζ nor CD3η is essential for IL-2 production when TCR complexes are strongly crosslinked by mAb. Given this result and the finding that no detectable Ca^{2+} mobilization was observed in MA5.8 cells after anti-CD3ϵ crosslinking, Ca^{2+} mobilization may not be essential for IL-2 production in MA5.8.

TABLE 1

IL2 Production of CD3ζ and CD3η Transfectants

IL2 production (units/ml/3 x 10^5 cells)

Expt. 1

	2B4.11	MA5.8	MAζ15.4	MAζ43.2	MAζ-η52.7	MAζ-η52.9	MAη61.3	MAη61.9
Ag(μM)								
0	<3	<3	<3	<3	<3	<3	<3	<3
4	45	<3	210	85	33	20	14	6
10	120	<3	500	380	75	60	50	30
25	300	<3	450	380	180	170	110	40

Exp. 2

	2B4.11	MA5.8	MAζ15.4	MAζ201	MAζ-η301	MAη61.9
Ag(μM)						
0	<4	<4	<4	<4	<4	<4
0.25	36	<4	45	30	90	<4
0.74	110	<4	80	53	130	8
2.2	150	<4	95	60	190	10
6.7	240	<4	100	70	240	12
20.0	240	<4	110	73	240	12
2C11 cross-linking(μg/well)						
0	<4	<4	<4	<4	<4	<4
0.04	22	<4	75	150	20	<4
0.4	520	90	230	460	330	20
4	520	90	270	460	330	20

While activation of T cells through the TCR results in tyrosine phosphorylation of the CD3ζ subunit (Baniyash, M. et al., J. Biol. Chem. 263:18225-18230 (1988)), it is unknown whether CD3η is similarly phosphorylated. These subunits may differ in their ability to serve as substrates for protein tyrosine kinase(s) (PTKs) and/or in their ability to activate PTK(s) upon TCR stimulation. CD3η lacks one of six potential tyrosine phosphorylation sites (Tyr 132) as well as a putative nucleotide binding site present in CD3ζ as a consequence of alternative splicing of the CD3ζη pre-mRNA (Weissman, A.M. et al., Science 239:1018-1021 (1988); Jin, Y-J. et al., Proc. Natl. Acad. Sci. USA 87:3319-3323 (1990)). To determine whether CD3η is phosphorylated upon TCR triggering, the tyrosine phosphorylation pattern of cellular proteins in MAζ15.4, MAζ-η301 and MAη61.9 was analyzed. MAζ-η301 cells were stimulated by TCR crosslinking with anti-Ti mAb. Subsequently, the TCR complex was immunoprecipitated from stimulated or unstimulated cells with anti-CD3ε mAb and analysed by western blotting with anti-phosphotyrosine mAb (anti-P-Tyr) 4G10 (Druker, B.J. et al., N. Eng. J. Med. 321:1383-1391 (1989)). Phosphorylation of a protein which migrates at 32KD in non-reducing SDS-PAGE was observed upon stimulation of MAζ-η301 via the TCR. This 32KD protein migrates as 21KD protein after reduction in 2-D diagonal gels, the known aberrant migration position of the phosphorylated CD3ζ subunit (Baniyash, M. et al., J. Biol. Chem. 263:18225-18230

(1988)). Since only one disulfide-linked phosphoprotein was observed in the TCR complex, the CD3ζ protein in $CD3\zeta_2$ but not in CD3ζ-η was detectably phosphorylated. In independent in vivo labeling studies, the CD3ζ protein in $CD3\zeta_2$ was also the only disulfide-linked phosphoprotein detected in anti-CD3ε immunoprecipitates from ^{32}P orthophosphate-labeled protein in MAζ-η301 cells following TCR stimulation. Furthermore, no phosphorylation of CD3η was detected by using the same method in MAη61.9 cells.

To verify the above findings, the tyrosine phosphorylation of cellular proteins in resting and TCR activated MAζ15.4, MAη61.9 and MAζ-η301 by immunoprecipitation and western blotting with anti-P-Tyr mAb was examined. Several proteins including an obvious ~82KD protein were phosphorylated upon TCR stimulation in each of the three transfectants. In contrast, a 32KD phosphoprotein was observed in activated MAζ15.4 and MAζ-η301 but not in activated MAη61.9. The 32KD protein in CD3ζ based on its migration in 2D-diagonal gels. Note that since phosphorylation of other proteins including the ~82KD band were observed in MAη61.9, the TCR isoform Tiα-βCD3γδεη-η was able to activate a PTK(s) responsible for their phosphorylation. These results demonstrate that CD3ζ but not CD3η was tyrosine phosphorylated upon TCR crosslinking and that all TCR isoforms can activate PTK(s) after stimulation. The fact that the CD3η subunit is not phosphorylated, yet contains five out of six tyrosine residues present in the

mature CD3ζ, strongly implies either that tyrosine 132 is the site of phosphorylation in CD3ζ or that the carboxy terminal CD3ζ segment is necessary to directly or indirectly mediate its association with a PTK. Furthermore, because only a single dimeric protein species phosphorylated upon stimulation in MAζ-η301 was observed and the 32KD band is the only apparent TCR-associated tyrosine phosphorylated protein observed in MAζ-η301 after 15 min, 30 min and 60 min stimulation, it appears that CD3ζ phosphorylation on tyrosine residues after TCR stimulation is restricted to the CD3ζ subunits of the $CD3\zeta_2$ homodimer. Since the level of $CD3\zeta_2$ dominates over CD3ζ-η and $CD3\eta_2$, it is difficult to formally exclude the possibility that a small fraction of CD3ζ subunits within the CD3ζ-η heterodimer are phosphorylated. However, this possibility is less likely in view of the substantial amount of CD3ζ-η heterodimer in MAζ-η301.

The clear differential tyrosine phosphorylation between CD3ζ and CD3η subunits stands in marked contrast to the ability of all CD3ζ/CD3η isoforms to transmit other early signals such as Ca^{2+} mobilization and PI turnover as well as the more complex responses required for the subsequent IL-2 gene activation upon antigen/MHC stimulation. Based on these results, it is unlikely that CD3ζ phosphorylation directs the primary signalling of these responses. However, given the regulatory role of tyrosine phosphorylation in many receptor systems (Hunter, T. and J.A. Cooper, Annu. Rev. Biochem.

54:897-930 (1985); Sibley, D.R. et al., Cell 48:913-922 (1987)), phosphorylation of CD3ζ could subsequently affect stimulation of these same pathways or modify other transduction signals. It will be important, for example, to determine whether CD3ζ phosphorylation desensitizes TCR triggering by antigen/MHC. If so, TCRs expressing the CD3ζ subunit will not be subjected to the same effects and, therefore, differential expression of TCR isoforms during T lineage development or in T cell subsets could qualitatively modify the cellular response. It has already been shown, for example, that in contrast to mature T cells, the CD3ζ subunit is constitutively phosphorylated on at least one of its tyrosine residues in thymocytes (Nakayama, T. et al., Nature 341:651-654 (1989)). Such a difference could account for functional distinctions in responsiveness of thymocytes v. mature T cells to TCR crosslinking as noted previously (Ramarli, D. et al., Proc. Natl. Acad. Sci. USA 84:8598-8602 (1987)).

Biological Deposit

E. coli strain XL1 Blue containing plasmid pBS17 was deposited on January 19, 1990, with the American Type Culture Collection (ATCC), Rockville, Maryland, according to the terms of the Budapest Treaty. The deposit has been assigned ATCC Accession Number 68206.

Equivalents

Those skilled in the art will recognize, or be able to ascertain employing no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 69, lines 22-28 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
19 January 1990	68206

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. 68206 shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

Charlotte E. Simon
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

CLAIMS

1. Isolated DNA of eukaryotic origin encoding CD3η T lymphocyte surface receptor subunit or a fragment thereof.

2. DNA of Claim 1 which is of rodent or human origin.

3. DNA of Claim 2, encoding all or a portion of the cytoplasmic domain of CD3η.

4. Isolated DNA having all or a portion of the nucleotide sequence of Figure 1, or a functional equivalent thereof.

5. A soluble CD3η T lymphocyte surface receptor subunit that corresponds to the extracellular domain of CD3η or a portion thereof.

6. A soluble CD3η T lymphocyte surface receptor subunit that corresponds to the cytoplasmic domain of CD3η or portion thereof.

7. A probe comprising DNA of eukaryotic origin encoding CD3η T lymphocyte surface receptor subunit or a fragment thereof.

8. A probe of Claim 7, wherein the DNA is of rodent or human origin.

9. A probe comprising DNA having all or a portion of the nucleotide sequence of Figure 1, or a functional equivalent thereof.

10. Essentially pure CD3η T lymphocyte surface receptor subunit homodimer of eukaryotic origin.

11. Essentially pure CD3η T lymphocyte surface receptor subunit homodimer encoded by all or a portion of the nucleotide sequence of Figure 1, or a functional equivalent thereof.

12. A method of detecting CD3η in T lymphocyte lineage cells in tissue, comprising:

a) obtaining DNA from the genome of cells to be assayed;

b) fragmenting the DNA into a multiplicity of DNA fragments;

c) hybridizing the DNA fragments with a probe containing nucleic acid sequences complementary to DNA encoding a CD3η T lymphocyte surface receptor subunit, under conditions which allow the probe to bind to DNA fragments having DNA sequences complementary to the CD3η DNA; and

d) detecting DNA fragments which are bound to the probe.

13. A method of Claim 12, wherein the probe comprises DNA having all or a portion of the sequence of Figure 1, or a functional equivalent thereof.

14. An expression vector having inserted therein isolated DNA as claimed in Claim 1.

15. An expression vector having inserted therein isolated DNA as claimed in Claim 4.

16. A cell transformed with the expression vector of Claim 15.

17. A monoclonal antibody directed against CD3η surface receptor subunit protein encoded by DNA having all or a portion of the sequence of Figure 1, or a functional equivalent thereof.

18. An immunochemical assay for detecting CD3η in T lymphocyte lineage cells in tissue, comprising:
a) obtaining a tissue sample to be assayed;
b) incubating the sample with antibody against CD3η surface receptor subunit protein; and
c) detecting complex formed between the antibody and the protein.

19. A method of screening for a CD3-mediated cellular response to a substance, comprising the steps of:
a) providing a cell which does not normally express CD3η subunit protein at significant levels, transformed with an expression vector containing CD3η encoding DNA or fragment thereof which causes the cell to express CD3η on its surface;
b) contacting the transformed cell with the substance under conditions which would permit the substance to complex with the CD3η subunit protein; and

c) measuring inositol phosphate concentration within the cell, whereby a change in concentration is indicative of CD3-mediated cellular response to the substance.

20. A method of screening for a substance that can block CD3 surface receptor function, comprising the steps of:

a) providing a cell which does not normally express CD3η subunit protein at significant levels, transformed with an expression vector containing CD3η encoding DNA or fragment thereof which causes the cell to express CD3η on its surface;

b) contacting the transformed cell with the substance and a CD3 activating agent, under conditions which would permit the substance and the CD3 activating agent to complex with the CD3η subunit protein; and

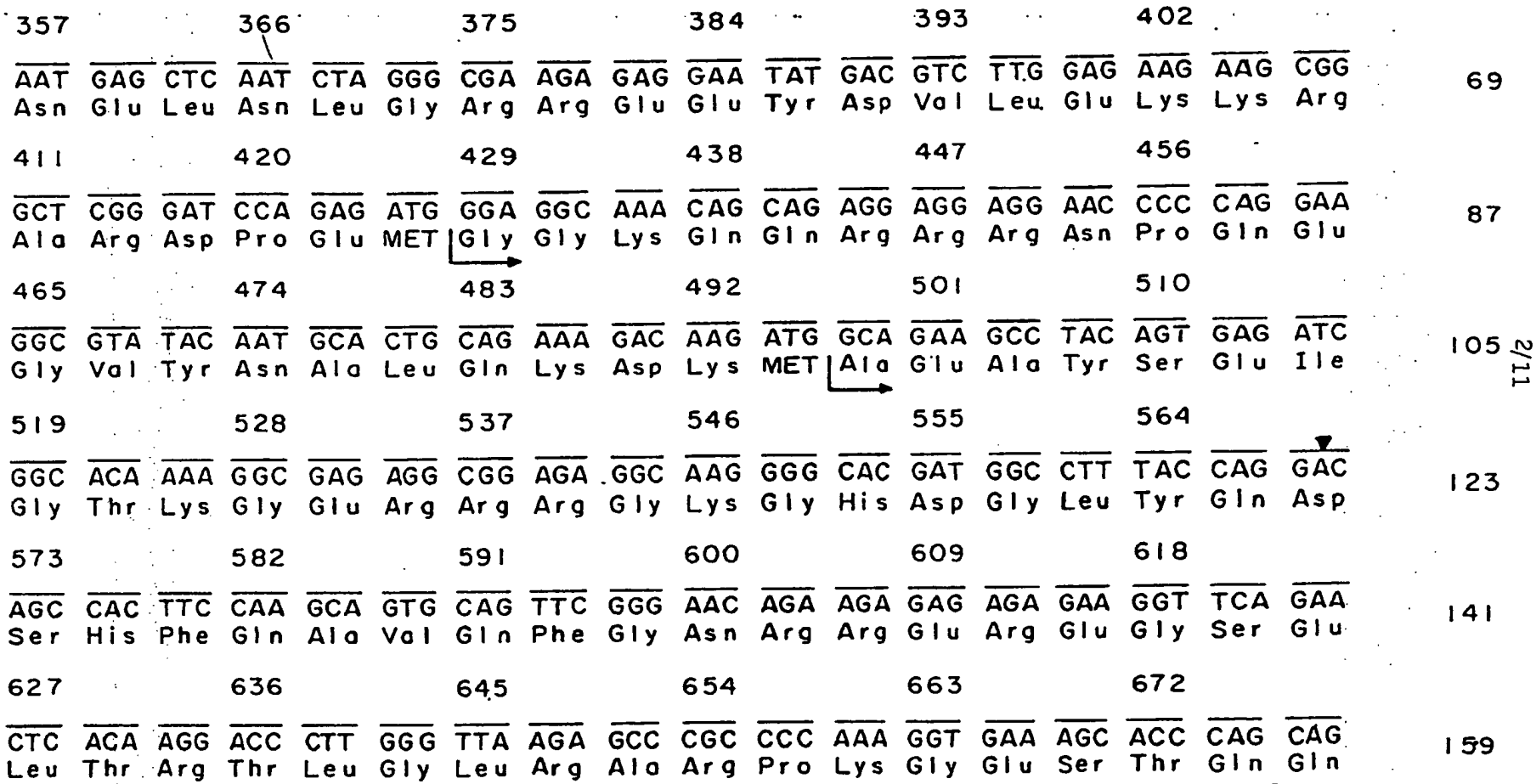
c) measuring inositol phosphate concentration within the cell, as indicative of blocking activity of the substance.

21. A soluble CD3η T lymphocyte surface receptor subunit that corresponds to the extracellular domain of CD3η or portion thereof, as defined in any one of Claims 5, 6, 10 or 11 , for use in medicine, for example in inhibiting T lymphocyte function.

22. The use of a soluble CD3η T lymphocyte surface receptor subunit that corresponds to the extracellular domain of CD3η or portion thereof, as defined in any one of Claims 5, 6, 10 or 11 , for the manufacture of a medicament for inhibiting T lymphocyte function.

```
        10          20          30          40          50          60          70
CGAGGAGAGG CACCCGGTAC ATGTTCTCTA GGAGAACAAA GCAAGCAGAG ACTCCATCAG CGCCTCCTTT

        80          90          100         110         120         130
TCTCCTCATC CTCCCAGGCA TAGCTGCCTC TGCCTCTGCC TCTGGGTACC ATCCCAGGGA AGCAGAAG

141         150         159         168         177         186
>
ATG AAG TGG AAA GTG TCT GTT CTC GCC TGC ATC CTC CAC GTG CGG TTC CCA GGA
Met Lys Trp Lys Val Ser Val Leu Ala Cys Ile Leu His Val Arg Phe Pro Gly      -4

195         204         213         222         231         240
GCA GAG GCA CAG AGC TTT GGT CTG CTG GAT CCC AAA CTC TGC TAC TTG CTA GAT
Ala Glu Ala Gln Ser Phe Gly Leu Leu Asp Pro Lys Leu Cys Tyr Leu Leu Asp      15

249         258         267         276         285         294
GGA ATC CTC TTC ATC TAC GGA GTC ATC ATC ACA GCC CTG TAC CTG AGA GCA AAA
Gly Ile Leu Phe Ile Tyr Gly Val Ile Ile Thr Ala Leu Tyr Leu Arg Ala Lys      33

303         312         321         330         339         348
TTC AGC AGG AGT GCA GAG ACT GCT GCC AAC CTG CAG GAC CCC AAC CAG CTC TAC
Phe Ser Arg Ser Ala Glu Thr Ala Ala Asn Leu Gln Asp Pro Asn Gln Leu Tyr      51
```

FIG. 1A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69
411 420 429 438 447 456
GCT CGG GAT CCA GAG ATG GGA GGC AAA CAG CAG AGG AGG AGG AAC CCC CAG GAA
Ala Arg Asp Pro Glu MET Gly Gly Lys Gln Gln Arg Arg Arg Asn Pro Gln Glu
87
465 474 483 492 501 510
GGC GTA TAC AAT GCA CTG CAG AAA GAC AAG ATG GCA GAA GCC TAC AGT GAG ATC
Gly Val Tyr Asn Ala Leu Gln Lys Asp Lys MET Ala Glu Ala Tyr Ser Glu Ile
105
519 528 537 546 555 564
GGC ACA AAA GGC GAG AGG CGG AGA GGC AAG GGG CAC GAT GGC CTT TAC CAG GAC
Gly Thr Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly Leu Tyr Gln Asp
123
573 582 591 600 609 618
AGC CAC TTC CAA GCA GTG CAG TTC GGG AAC AGA AGA GAG AGA GAA GGT TCA GAA
Ser His Phe Gln Ala Val Gln Phe Gly Asn Arg Arg Glu Arg Glu Gly Ser Glu
141
627 636 645 654 663 672
CTC ACA AGG ACC CTT GGG TTA AGA GCC CGC CCC AAA GGT GAA AGC ACC CAG CAG
Leu Thr Arg Thr Leu Gly Leu Arg Ala Arg Pro Lys Gly Glu Ser Thr Gln Gln
159

FIG. 1B

```
681          690          699          708          717          726
AGT AGC CAA TCC TGT GCC AGC GTC TTC AGC ATC CCC ACT CTG TGG AGT CCA TGG      177
Ser Ser Gin Ser (Cys) Ala Ser Val Phe Ser Ile Pro Thr Leu Trp Ser Pro Trp

735          744          753          766          776          786          796
CCA CCC AGT AGC AGC TCC CAG CTC> TAAGGCCCTG TGCTCAGCTC TGGTGATGAC CCTGGCTGCT  185
Pro Pro Ser Ser Ser Ser Gin Leu

        806          816          826          836          846          856          866
GTCACATGAG TTGTGGTGAG GATGGGACTT TTGAAAAATC TGATGTTCCA ATTTCTTTCA TGCATGCTCT

        876          886          896          906          916          926          936
ACTCAGAAGT GAGCAAGGGC CAAAACTCCT GGGCATGCAT CTGAATGAAT CTCTCAACTT AGAAAAGACT

        946          956          966          976          986          996          1006
TGCCTGCCCC TCTGAGCTGG CCAGGTGTCC CCACCTACCC TTTGGCATGC CTCCAAGTGC CAGGACGCCA
```

FIG. 1C

1016 1026 1036 1046 1056 1066 1076

CAGACTGCAT TGGGGCCGAA GACTTCCCTT TTCTTTGGTT TTTTTGTTTG TTTTGAGTTT ATACAATCAT

1086 1096 1106 1116 1126 1136 1146

TAAGAAATCT TTGGTTTTGG CTGGAAATGG AAAAACAAAA CAAATCAAAG AAACAACCCT CCCCTGGCTT

1156 1166 1176 1186 1196 1206 1216

ATAGCAGCAG TATTATGACC TGACCTGGCT GAGCTTTCCC CCCCTCCAAC TTTGGGGGTC GAAATTGCAA

1226 1236 1246 1256 1266 1276 1286

GTTAAGAACT ACATTCAAAG AAAACGTTGA AAGGGCCGGA GAAGCAGCTT CCAGAAAGCC CGTCGGATAT

1296 1306 1316 1326 1336 1346 1356

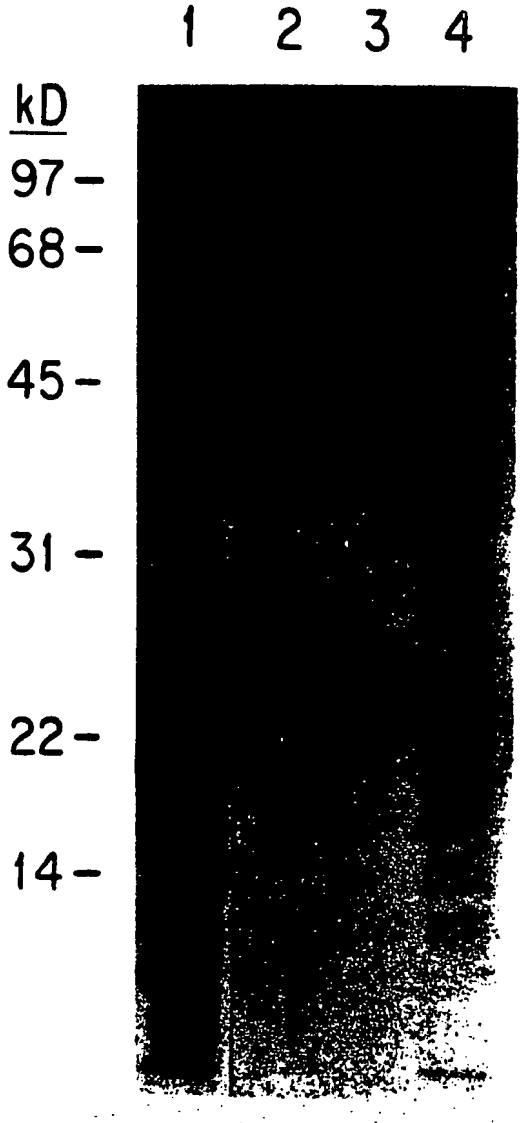
AAGATTGTCA AATAATAATA ACTATTATTA TAATATATTA ATAATAAACT TAAATATTTG GAAAAAAAAA...

FIG. 1d

```
Eta     QSFGLLDPKLCYLLDGILFIYGVIITALYLRAKFSRSAETAANLQDPNQL -50
        :::::::::::::::::::::::::::::::::::::::::::::::::
Zeta    QSFGLLDPKLCYLLDGILFIYGVIITALYLRAKFSRSAETAANLQDPNQL -50

Eta     YNELNLGRREEYDVLEKKRARDPEMGGKQQRRRNPQEGVYNALQKDKMAE -100
        :::::::::::::::::::::::::::::::::::::::::::::::::
Zeta    YNELNLGRREEYDVLEKKRARDPEMGGKQQRRRNPQEGVYNALQKDKMAE -100

Eta     AYSEIGTKGERRRGKGHDGLYQDSHFQAVQFGNRREREGSELTRTLGLRA -150
        :::::::::::::::::::::
Zeta    AYSEIGTKGERRRGKGHDGLYQGLSTATKDTYD---------------- -133

Eta     RPKGESTQQSSQSCASVFSIPTLWSPWPPSSSSQL -185
Zeta    ------------------------------ALHMQTLAPR -143
```

FIGURE 2

FIG.3



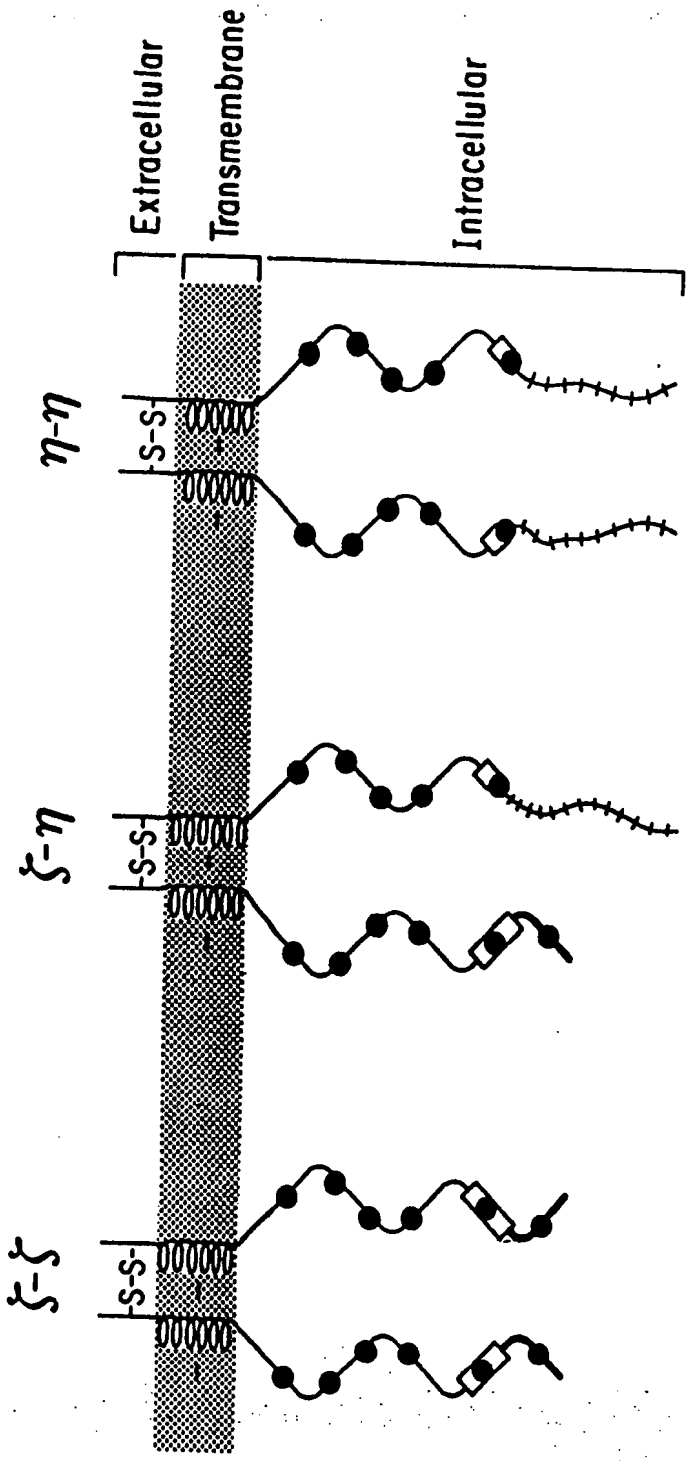

Extracellular
Transmembrane
Intracellular
η-η
ζ-η
ζ-ζ
S-S
S-S
S-S

FIG. 4

FIG.5

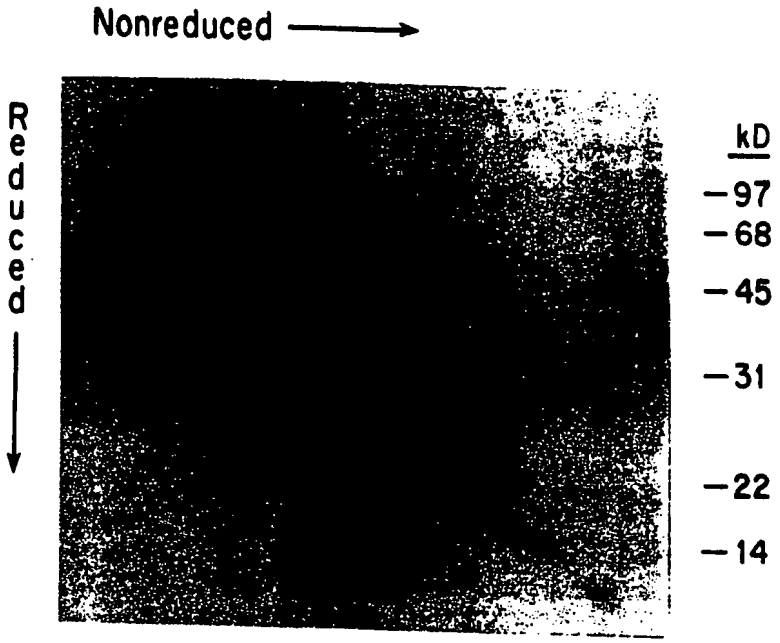
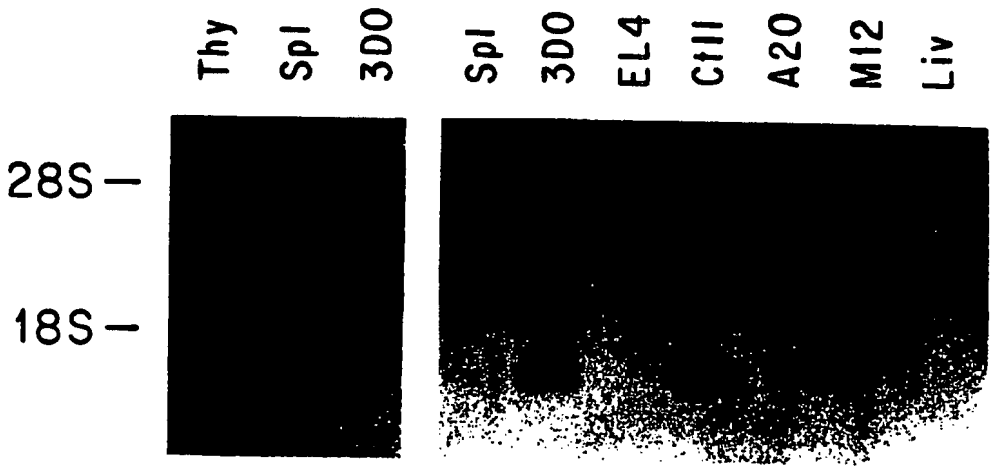


FIG.6a FIG.6b



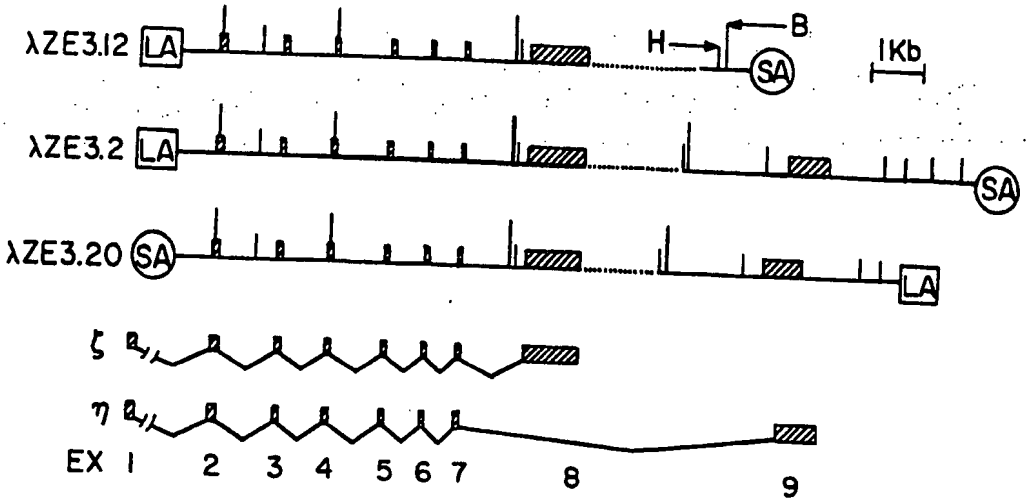

λZE3.12
LA
H
B
SA
1Kb
λZE3.2
LA
SA
λZE3.20
SA
LA
ζ
η
EX 1 2 3 4 5 6 7 8 9

FIG. 7

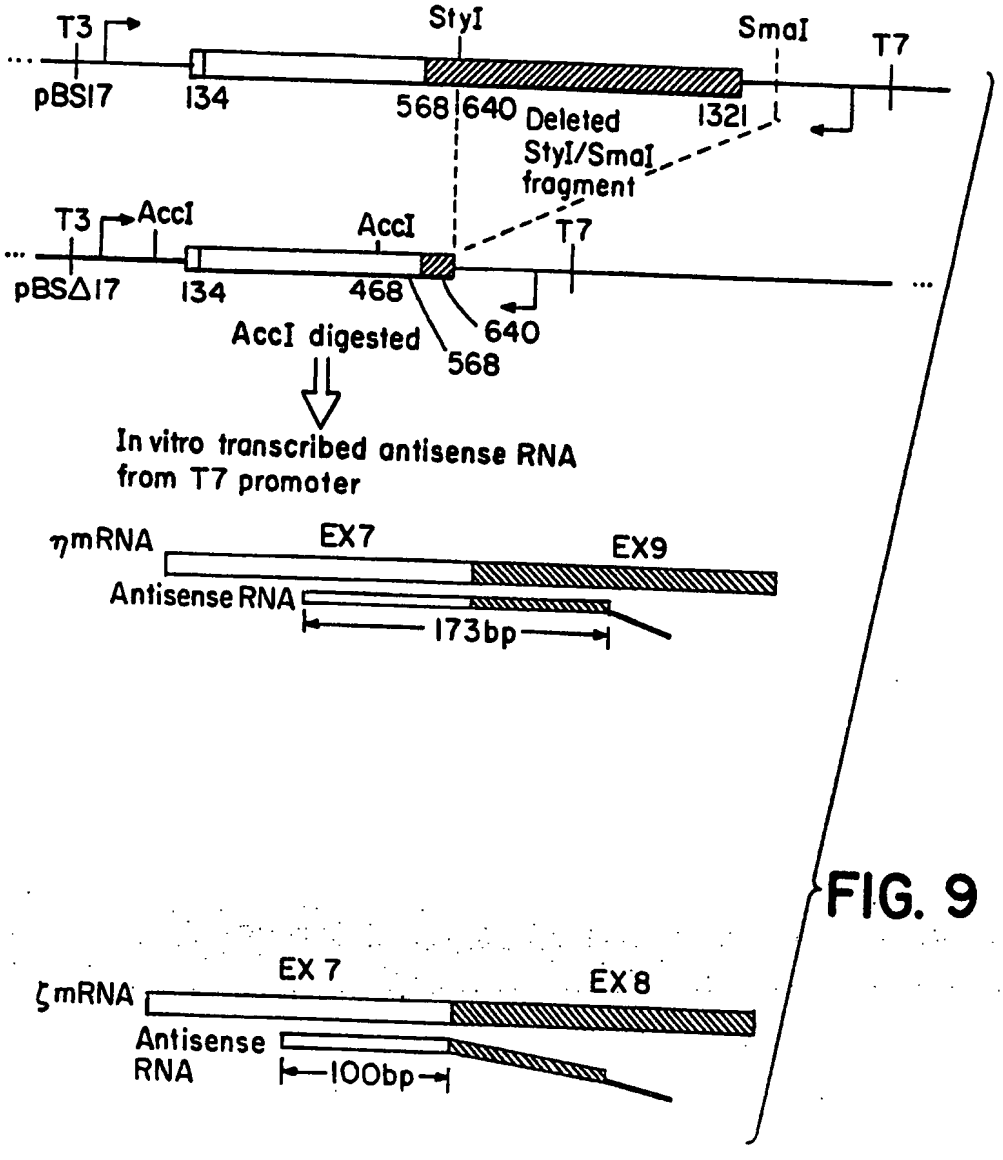

T3
StyI
SmaI
T7
pBS17
134
568
640
Deleted
StyI/SmaI
fragment
1321
T3
AccI
AccI
T7
pBSΔ17
134
468
640
568
AccI digested
In vitro transcribed antisense RNA
from T7 promoter
ηmRNA
EX7
EX9
Antisense RNA
173bp
ζmRNA
EX 7
EX 8
Antisense
RNA
100bp

FIG. 9

..tgtttcctcacaaacagGAC AGC CAC TTC CAA GCA GTG CAG TTC GGG AAC AGA AGA
Asp Ser His Phe Gln Ala Val Gln Phe Gly Asn Arg Arg

GAG AGA GAA GGT TCA GAA CTC ACA AGG ACC CTT GGG TTA AGA GCC CGC CCC AAA
Glu Arg Glu Gly Ser Glu Leu Thr Arg Thr Leu Gly Leu Arg Ala Arg Pro Lys

GGT GAA AGC ACC CAG CAG AGT AGC CAA TCC TGT GCC AGC GTC TTC AGC ATC CCC
Gly Glu Ser Thr Gln Gln Ser Ser Gln Ser Cys Ala Ser Val Phe Ser Ile Pro

ACT CTG TGG AGT CCA TGG CCA CCC AGT AGC AGC TCC CAG CTC TAA GGCCCTGTGCT
Thr Leu Trp Ser Pro Trp Pro Pro Ser Ser Ser Ser Gln Leu stop

CAGCTCTGGTGATGACCCTGGCTGCTGTCACATGAGTTGTGGTGAGGATGGGACTTTTGAAAAATCTGATGT

TCCAATTTCTTTCATGCATGCTCTACTCAGAAGTGAGCAAGGGCCAAAACTCCTGGGCATGCATCTGAATGA

ATCTCTCAACTTAGAAAAGACTTGCCTGCCCCTCTGAGCTGGCCAGGTGTCCCCACCTACCCTTTGGCATGC

CTCCAAGTGCCAGGACGCCACAGACTGCATTGGGGCCGAAGACTTCCCTTTTCTTTGGTTTTTTTGTTTGTT

TTGAGTTTATACAATCATTAAGAAATCTTTGGTTTTGGCTGGAAATGGAAAAACAAAACAAATCAAAGAAAC

CACCCTCCCCTGGCTTATAGCAGCAGTATTATGACCTGACCTGGCTGAGCTTTCCCCCCCTCCAACTTTGGG

GGTGGAAATTGCAAGTTAAGAACTACATTCAAAGAAAACGTTGAAAGGGCCGGAGAAGCAGCTTCCAGAAAG

CCCGTCGGATATAAGATTGTCAAATAATAATAACTATTATTATAATATATTAATAATAAACTTAAATATTTG

GAAttcttttggtcat...

FIGURE 8